JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1983, p. 202-217
0095-1137/83/020202-16$02.00/0

Vol. 17, No. 2

Evaluation of a Computer-Assisted, Kinetics-Based Enzyme-Linked Immunosorbent Assay for Detection of Coronavirus Antibodies in Cats

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Received 21 July 1982/Accepted 1 November 1982

A computer-assisted, kinetics-based enzyme-linked immunosorbent assay was adapted for the detection of coronavirus antibodies in feline serum. An alkaline antigen diluent (carbonate-bicarbonate buffer, pH 9.6) used in initial experiments produced diffuse, nonspecific color reactions in both viral and control antigen cuvettes which were correlated, paradoxically, with coronavirus antibody levels in test sera. These interfering reactions were minimized by use of lower-pH antigen diluents such as water and phosphate-buffered saline. Background kinetics-based enzyme-linked immunosorbent assay reactivity directed against a noncoronaviral component of antigen tissue culture fluids could then be detected in numerous sera, particularly in samples with lower titers. Much of this reactivity was shown to be associated with bovine gamma globulins in cell culture fluid. It was not serum lot or species specific, since a variety of bovine serum lots as well as individual lots of serum from other mammalian and avian species reacted. Reactivity was markedly reduced when cells for antigen preparation were grown in gamma globulin-free bovine serum. Generation of corrected slope values from the kinetics-based enzyme-linked immunosorbent assay made it possible to correct for residual background reactivity in individual test sera and thus eliminate a potentially major source of false-positive reactions. Collectively, these studies indicated that the control of nonspecific reactivity in feline coronavirus serology is absolutely essential to obtain useful estimates of specific antibody responses.

The coronaviruses are a closely related family of single-stranded RNA viruses and are important causes of upper respiratory and enteric disease, hepatitis, serositis, and encephalitis in several species of birds and mammals (6, 52, 62). In cats the feline infectious peritonitis virus is the etiological agent of a lethal disease involving widespread perivascular pyogranulomatous reactions found most commonly within the serosal membranes, liver, kidney, omentum, lung, eyes, and central nervous system (16, 31, 39, 71). These reactions are the result of immunologically mediated phenomena involving Arthus-like antigen-antibody-complement interactions across vessel walls (21, 42, 66–69). Recently, several reports of coronaviruses localized to the gastrointestinal tract of cats have also appeared (11, 17, 20, 28, 43). Some of these agents appear to be antigenically similar (if not identical) to feline infectious peritonitis virus (17, 28, 43).

The humoral immune response of cats to a coronavirus(es) has been studied by using several techniques, including indirect immunofluorescence assay (IFA) (26, 35, 40, 48), enzyme-linked immunosorbent assay (ELISA) (36, 41), virus neutralization (19, 28, 49, 50, 60), and passive hemagglutination (60). Because feline infectious peritonitis virus has until recently been impossible to propagate in conventional monolayer cell cultures, most of these assays have relied upon the antigenic relationship which exists between feline infectious peritonitis virus and certain less fastidious coronaviruses, such as transmissible gastroenteritis virus (TGEV) of swine, canine coronavirus (CCV), and human respiratory coronavirus 229E (35, 44, 49, 51, 70). Although IFA is the only one of these procedures to have been widely utilized thus far in clinical veterinary serology, ELISA has shown excellent potential as a rapid and sensitive method for detection of coronavirus antibodies in cats (2, 36).

Although the introduction of enzyme immunoassays such as ELISA for the determination and

quantitation of soluble constituents (analytes) has offered new serodiagnostic potential to the medical and veterinary medical sciences, it has become apparent that certain deficiencies of conventional ELISA methodology have not been adequately addressed. For example, error may be introduced when correlation of different serological assay titers to ELISA absorbance readings is attempted without regard for the kinetics of the enzyme-substrate reaction. If the ELISA is read after the enzyme-substrate reaction rate has exceeded its linear limits, then correlation of absorbance with other serological assay titers cannot be justified. In addition, conventional ELISA titers are often dependent upon end-point determinations in the presence of stopping reagents which may not completely halt chromogen color shifts (8). A third drawback of some ELISA methodologies is the requirement for serial dilutions of test serum. When the ELISA is based on enzyme-substrate reaction rate kinetics, however, these disadvantages are circumvented, and linear quantitative data are produced which can be readily converted to a continuous scale of titers (61). Unfortunately, insufficient effort has been made to date toward development of inexpensive kinetics-based immunoassay systems which are capable of a high sample throughput and provide for rapid and accurate detection of analytes.

By utilizing computer-assisted semiautomated immunoassay technology, a test system has been developed at the New York State Diagnostic Laboratory (NYSDL) for quantitation of analytes which is suitable for high-throughput applications and reduces to a minimum numerous sources of error that are common to other immunoassay systems (21a). In this kinetics-based ELISA (KELA), the enzyme-substrate reaction rate is determined and is directly proportional to the quantity of analyte in the sample. Although a computer-assisted KELA might suggest a highly complicated assay system, appropriate software has been developed in the Automated Serology Laboratory of the NYSDL which minimizes physical manipulations and provides for maximum utilization of computer technology.

In this paper we report the adaptation of the KELA system to detection of coronavirus antibodies in feline serum.

(This work was performed in partial fulfillment of the requirements for a Ph.D. thesis, Cornell University, Ithaca, N.Y., by J.E.B.)

MATERIALS AND METHODS

Virus stocks. The Miller strain of TGEV was obtained from National Veterinary Services Laboratories, Ames, Iowa. The 1-71 strain of CCV was obtained from L. E. Carmichael of the James A. Baker Institute for Animal Health, Cornell University. Viruses were initially grown in secondary canine kidney cells, frozen, and thawed, and the supernatant fluids obtained after low-speed centrifugation were frozen in working volumes at −90°C.

Cell cultures. Both viruses were propagated in canine A-72 cells (3). The growth medium consisted of Eagle minimum essential medium containing Earle salts and L-glutamine (GIBCO Laboratories, Grand Island, N.Y.), 0.05% lactalbumin hydrolysate (GIBCO), 100 U of potassium penicillin G per ml, 100 μg of streptomycin sulfate per ml, 50 μg of gentamicin sulfate per ml, 2.5 μg of amphotericin B per ml, and sodium bicarbonate. The medium was supplemented with either 10% newborn calf serum (NCS) (GIBCO, lot no. 25K5004) or 10% gamma globulin-free NCS (GGF-NCS) (GIBCO, lot no. C194419).

Viral and control antigen preparations. At 48 h after virus adsorption, flasks were subjected to one cycle of freezing and thawing, and the tissue culture fluids were centrifuged at 2,000 × *g* for 20 min at 4°C. Supernatant fluids were pooled and stored in samples at −75°C. Virus titers of the TGEV preparations grown in 10% NCS and in 10% GGF-NCS were 4.48×10^5 and 4.68×10^5 PFU/ml, respectively, as assayed on secondary feline kidney cells. The titer of the CCV preparation grown in 10% NCS was 1.71×10^5 PFU/ml, as assayed on A-72 cells. Control cells were grown appropriately in either NCS or GGF-NCS and treated in an identical manner except that virus was omitted.

For differential centrifugation, cell culture fluids were sonicated four times for a total of 60 s with a model 150 sonic dismembrator (Artek Systems Corp., Farmingdale, N.Y.) and centrifuged (15,000 × *g*) for 30 min at 4°C. Supernatant fluids were then centrifuged again at 60,000 × *g* for 60 min. The resulting pellets were suspended in 2 ml of STE buffer (0.1 M NaCl, 0.01 M Tris, 1 mM disodium EDTA, pH 7.5) and incubated overnight at 4°C. Preparations were then centrifuged at 15,000 × *g* for 30 min, and the supernatant fluids were recentrifuged at 90,000 × *g* for 60 min. Pellets were resuspended in STE buffer and again incubated overnight at 4°C. Preparations were then spun at 15,000 × *g* for 30 min, and the supernatant fluids were recentrifuged at 135,000 × *g* for 60 min. Final pellets were suspended in 2 ml of 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6), incubated overnight at 4°C, dispensed (undiluted) in 200-μl amounts into 750-μl cuvettes in polystyrene-copolymer EIA cuvette packs (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), and dried in an unhumidified incubator (37°C) for 36 to 40 h.

Feline sera. Serum samples used to evaluate the KELA were obtained from a variety of sources. The majority were submitted to the NYSDL by private veterinary practitioners requesting IFA titers for coronavirus antibodies. A smaller number of sera were submitted by clinicians in the small animal clinic, Veterinary Medical Teaching Hospital, Cornell University. Other sera were obtained from a minimal-disease feline breeding colony maintained by the Division of Laboratory Animal Services, Cornell University, and from minimal-disease kittens purchased from a commercial breeding colony (Liberty Laboratories, Liberty Corner, N.J.) and maintained in isolation. Parallel IFA titers for coronavirus antibodies were

performed on all sera evaluated by the KELA.

Indirect IFA. The Miller strain of TGEV was grown in either secondary canine kidney cells or A-72 cells, and coronavirus antibody assays were performed by using either glass microscope slides or disposable plastic Leighton tubes (Costar, Cambridge, Mass.), as previously described (48). Test sera were diluted 1:5, 1:25, 1:100, 1:400, and 1:1,600. Samples without specific viral fluorescence at a dilution of 1:5 were considered negative (<1:5). A group of sera was also identified which showed elevated levels of background fluorescence at low to moderate dilutions, making positive identification of virus-specific fluorescence difficult. These sera were designated ≤1:25 and considered separately in field survey evaluations.

KELA antigen preparations. To prepare tissue culture fluids for the KELA, samples of viral and control preparations were quickly thawed and diluted 1:10, 1:50, 1:100, or 1:1,000 in one or more of the following diluents: (i) 0.1 M carbonate-bicarbonate buffer (pH 9.6), (ii) 0.1 M phosphate-buffered saline (PBS) (pH 7.4), (iii) double-distilled water (pH 7.0), and (iv) deionized water (pH 6.3). All diluents were sterilized and adjusted to 4°C before addition of tissue culture fluids. Diluted KELA antigen preparations were dispensed in 200-μl quantities into EIA cuvettes and dried at 37°C for 36 to 40 h. Coated packs were stored in sealed plastic bags at 4°C and used within a few days of preparation.

Enzyme-antibody conjugate. The immunoglobulin G (IgG) fraction of rabbit antiserum against feline IgG (light and heavy chains) conjugated to peroxidase (Cappel Laboratories, Cochranville, Pa.) was reconstituted with 1 ml of sterile deionized water, diluted 1:10 in 0.1 M PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) (PBST), and stored in samples at −20°C. The working dilution of this lot of conjugate was either 1:3,500 or 1:1,000 in PBST.

Substrate solution. A 1:20 dilution of H_2O_2 in sterile distilled water was further diluted 1:66 in a solution containing 50 mM citric acid (adjusted to pH 4.0 with 5 M NaOH immediately before use) and 0.4 to 1.6 mM 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (Sigma). The molarity of the stock H_2O_2 was checked periodically by absorption at 240 nm (54). Optimum activity was found between 8 and 11 M; solutions with molarity below 8 were discarded. The substrate solution was prepared immediately before use and shielded from light with aluminum foil.

Processing of samples. The KELA was developed by using semiautomated EIA System 50 technology (Gilford). The basic parameters of the KELA were determined, and preliminary field surveys were conducted, with this basic system under manual operator control. Optimization of the KELA protocol and processing of large numbers of NYSDL submission sera on a routine basis were carried out with computer assistance in the Automated Serology Laboratory of the NYSDL, where two EIA-PR50 processor units were fully interfaced with a PDP 11/34A computer (Digital Equipment Corp., Maynard, Mass.). Data points (absorbance readings at 405 nm) were transmitted automatically in ASCII format and stored for later retrieval and statistical analysis. Concurrently, a paper tape printout was provided as a backup in the event of computer malfunction. The processor units were placed in tandem positions so that EIA cuvette packs could be subjected to one treatment (e.g., dispensing of conjugate) sequentially and then moved automatically along a connecting bridge to the second processor for a subsequent treatment (e.g., aspiration of conjugate and dispensing of PBST wash solution). Tandem operation of EIA-PR50 units under computer control thus made it possible to process large numbers of samples per run (>150 samples per h). A cathode ray tube terminal was used as a control terminal for operation of the instruments and input of sample identifications.

KELA. The rate of reaction between bound peroxidase and substrate was determined by recording three absorbance readings at 4- or 5-min intervals, beginning 3 to 5 min after the addition of substrate solution. These intervals provided a linear relationship between absorbance values and time so that the resulting sample regression coefficient, or slope (representing the rate of substrate conversion by enzyme), was directly proportional to the quantity of analyte (i.e., coronavirus antibody) present in the sample (21a). Thus, the steeper the slope of the reaction curve, the higher the titer of coronavirus antibody. This approach provided a slope value which was independent of minor aberrations in cuvette walls which might affect absorbance readings and of the time interval between substrate addition and the first absorbance determination (a variable which, at least in our hands, has been difficult to properly control in massive screening procedures with the Gilford EIA System 50).

Computer assistance was found to be invaluable for high-throughput operation of the KELA. A sequencing program was developed by two of us (D.R.D. and T.J.L.) in which EIA-PR50 mode selection and operation were placed under direct computer control. A set of directives was provided on the cathode ray tube terminal for processing a predesignated number of EIA cuvette packs through the steps of the assay. The program directed continual running of packs by staggering the washing, reagent addition, and absorbance-reading steps while maintaining precise, predetermined intervals between all steps in the assay for each cuvette. In addition, the computer provided monitoring and analysis of reagent controls, including (i) efficiency of antigen binding to the solid phase, (ii) conjugate binding efficiency and reactivity (conjugate control), (iii) substrate solution stability, and (iv) intra-assay drift. The computer was also essential to (i) collect individual data points, (ii) establish and record time intervals between successive absorbance readings, (iii) calculate a slope and coefficient of determination (r^2, an indication of slope linearity) (56) for each sample, (iv) convert slope values to quantities of analyte via interpolation from a nomograph (calculated from known high and intermediate positive and negative serum controls normalized to expected values) (manuscript in preparation), and (v) provide printed reports of raw data points, slope and r^2 values, and mean ± standard deviation as well as coefficient of variation of sample and reagent control replicates. Uncorrected slope values and r^2 determinations were generated by the computer according to the following standard formulas (56):

$$\text{slope} = \frac{\Sigma(X \cdot Y) - (\Sigma X \cdot \Sigma Y)/n}{\Sigma X^2 - (\Sigma X)^2/n} \text{ and}$$

$$r^2 = \frac{[\text{slope}]\,[\Sigma(X \cdot Y) - (\Sigma X \cdot \Sigma Y)/n],}{\Sigma Y^2 - (\Sigma Y)^2/n},$$

where X = reading time in seconds, Y = absorbance value at 405 nm, and n = number of absorbance readings taken per sample (n = 3). Corrected slope values were then calculated as indicated in the legend to Fig. 1.

KELA protocols. Three protocols were investigated for detecting coronavirus antibodies (Table 1). All runs with antigen preparations diluted in carbonate-bicarbonate buffer followed protocol 1, which contained a prewash sequence. Initial runs with the other antigen diluents followed protocol 2. Because prewashing of the latter preparations resulted in reduced KELA reactivity when compared with that in unwashed cuvettes, the prewash sequence was deleted. Protocol 3 was an adaptation of protocol 2 to computer assistance after optimization. Various antigen, serum, and conjugate concentrations were tested with each protocol, and multiple replicates were performed on all samples to monitor within-run variation.

Preparation of goat IgG. Serum was obtained from a minimal-disease female goat maintained by the Department of Microbiology, Cornell University. The IgG fraction was separated by 50% ammonium sulfate precipitation and DEAE-Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) column chromatography. The purity of the IgG preparation was checked by immunoelectrophoresis in 1.5% agarose gels.

RESULTS

Preliminary experiments. In initial experiments, viral and control antigen preparations were diluted in carbonate-bicarbonate buffer and evaluated (protocol 1) with feline sera known to be positive for coronavirus antibodies by IFA. With CCV as the antigen, discrimination among levels of coronavirus antibody positivity was readily demonstrated; however, control antigen preparations were just as efficient at discriminating antibody levels as were CCV antigen preparations (Table 2). A visual inspection of cuvettes after the addition of substrate solution revealed development of a diffuse green color which originated within the solution itself rather than on the cuvette walls as expected; this reaction did not occur in empty (uncoated) cuvettes. Similar results were obtained with all sera tested and also with TGEV as the antigen (data not shown). These data suggested that the observed reactions were not wholly specific for coronavirus antibodies, but did not suggest an explanation for the broad titer discrimination obtained.

To determine whether pelletable material still present in tissue culture fluids might be responsible for these results, TGEV and control antigen tissue culture fluids were subjected to a differential centrifugation procedure and tested against a serum of high coronavirus antibody titer (IFA titer = 1:1,600). Discrimination between the two antigen preparations was slightly improved, but KELA slopes for the control cuvettes were still unacceptably high, and the reactions were again distributed diffusely throughout the substrate solution (data not shown).

These results suggested reactivity associated with some more general property of the antigen preparations, such as diluent buffer composition, ionic strength, or pH. Accordingly, experiments were devised to investigate the effects of different diluents on the assay. Tissue culture fluids were diluted in 0.1 M PBS (pH 7.4), double-distilled water (pH 7.0), or deionized water (pH 6.3) and compared with dilutions in carbonate-bicarbonate buffer with KELA protocol 2. Good discrimination between viral and control antigen preparations, with extremely low KELA slopes in control cuvettes, was obtained with all three diluents with a 1:50 test serum dilution (Table 3). Antigens diluted 1:10 in deionized water produced the widest range of discrimination. These reactions were observed to originate on the cuvette walls, with gradual diffusion of the colored product into the medium, suggesting specific activity involving antigen bound to polystyrene-copolymer. Carbonate-bicarbonate buffer thus appeared to be an inappropriate antigen diluent for this assay. Subsequent experiments suggested that high pH of the diluent was more important than ionic composition in producing the diffuse, nonspecific color reaction (data not shown). Because of its broader range of discrimination, deionized water was chosen as the antigen diluent for all further KELA runs. To fully evaluate its efficacy with a wide variety of test samples, a preliminary field survey with NYSDL submission sera was performed.

Preliminary field survey. A total of 324 individual feline serum samples, collected consecutively from private practitioner and Veterinary Medical Teaching Hospital clinician submissions to the NYSDL, were evaluated with either TGEV or CCV as the antigen (KELA protocol 2). A total of 59 of these sera were designated $\leq$1:25 by IFA, and their KELA slopes were considered separately.

A general correlation was obtained between uncorrected KELA slope and IFA titer with either TGEV (Fig. 1) or CCV (data not shown). Several sera had uncorrected KELA slopes that were much greater than expected, whereas the mean slope value for sera with titers of 1:5 was lower than that for <1:5 samples, so that titer discrimination at low slope values was poor. Plotting of corrected KELA slopes for these same sera produced a relative decrease in the mean slope value for the <1:5 sera by reducing many of the unexpectedly elevated slopes (Fig.

TABLE 1. KELA protocols evaluated for detection of coronavirus antibodies in feline serum[a]

Protocol	Cuvette prewash (×3)	Serum dilution (in PBST)	Serum dispensation (μl/cuvette)	Serum incubation[b] (min at 37°C)	Serum wash	Conjugate dilution (in PBST)	Conjugate dispensation[c] (μl/cuvette)	Conjugate incubation[c] (min at RT)	Conjugate wash[c]	Substrate solution dispensation[c] (μl/cuvette)	Absorbance reading[c] (405 nm) at three time intervals
1[d]	Rinse and fill; incubate at RT for 3 min; rinse and aspirate; invert and tap dry	1:100	200	20	Repeat prewash	1:3,500	100	30	Repeat prewash	400	Manual
2[e]	ND	1:50	100	20	As in protocol 1	1:3,500	100	30	As in protocol 1	400	Manual
3[e]	ND	1:100	100	45	Rinse and fill; incubate at RT for 10 min; rinse and aspirate; invert and tap dry	1:1,000	100	30	Repeat serum wash	400 (computer-controlled dispensation)	Computer controlled

[a] The steps of the KELA procedure are performed in order from left to right. Abbreviations: RT, room temperature; ND, not done.
[b] Performed in humidified incubator.
[c] Performed by computer sequencing program for protocol 3 only.
[d] Antigen dilution, 1:1,000 (200 μl per cuvette).
[e] Antigen dilution, 1:10 (200 μl per cuvette).

TABLE 2. Comparison of KELA slopes in the presence and absence of CCV antigen with selected feline sera[a]

Test sample	Coronavirus antibody titer[b]	KELA slope[c] (×10³) with:	
		Viral antigen[d]	Control antigen[e]
1	1:1600	160	167
2	1:400	58	66
3	<1:5	14	13
4[f]		7	8
5[g]		6	5

[a] Representative experiment.
[b] Determined by indirect IFA with TGEV in secondary canine kidney cells as the antigen.
[c] Determined with KELA protocol 1.
[d] Prepared from CCV-infected A-72 cell tissue culture fluid.
[e] Prepared from mock-infected A-72 cell tissue culture fluid.
[f] Antigen control (test serum omitted).
[g] Cuvette control (antigen coating and test serum omitted).

1). However, there was no improvement in titer discrimination at the lower end, and corrected slopes for many sera with higher titers were much lower than expected (data not shown).

Uncorrected KELA slopes (×10³) for sera designated ≤1:25 by IFA varied from 6 to 40 (Fig. 2), a range generally corresponding to titers of <1:5 to 1:100. Corrected KELA slopes tended to cluster around zero, suggesting that many of these sera were actually negative for coronavirus antibodies. Sera unequivocally negative by IFA also demonstrated this tendency (Fig. 1). Equivalent results were obtained when CCV was used as the antigen (data not shown).

Taken together, these data suggested the possibility (among others) that a non-coronaviral factor or factors present in both viral and control antigen tissue culture fluids might be contributing to variable alterations of selected IFA titers or KELA slopes or both and might also be responsible for difficulties in IFA interpretation of ≤1:25 sera. Accordingly, experiments were designed to further investigate this possibility.

Investigation of non-coronaviral reactivity in tissue culture fluid supernatants. To identify the source of the postulated non-coronaviral reactivity in tissue culture fluids, individual components of the fluid were diluted in deionized water, and 200-μl volumes were dried onto cuvettes. Each component was appropriately diluted so that its final concentration was equivalent to that in tissue culture fluid after a preparatory 1:10 dilution in deionized water. Serum samples were also tested against empty cuvettes to determine whether there might be reactivity in the absence of antigen. Both ≤1:25 and low-background sera were evaluated, using KELA protocol 2.

The majority of the non-coronaviral reactivity was found in bovine serum (Fig. 3). Most ≤1:25 sera reacted with both fetal bovine serum (FBS) and NCS, but in most cases the KELA slope for NCS was greater than that for FBS. In addition, two sera were identified which produced consistently elevated slope values with virtually all tissue culture fluid components; both of these

TABLE 3. Comparison of KELA slopes in the presence and absence of TGEV antigen with selected antigen diluents

Antigen diluent	Antigen dilution	KELA slope[a] (×10³) at the following test sample dilution[b]:			
		1:50		1:100	
		Viral antigen[c]	Control antigen[d]	Viral antigen	Control antigen
Carbonate-bicarbonate buffer (pH 9.6)	1:1,000	ND[e]	ND	63	51
PBS (pH 7.4)	1:100	23	13	14	10
	1:50	47	15	26	11
	1:10	72	15	24	10
Distilled water (pH 7.0)	1:100	ND	ND	21	6
	1:50	50	12	19	8
	1:10	63	10	19	7
Deionized water (pH 6.3)	1:10	113	19	ND	ND

[a] Determined with KELA protocol 2. KELA slope (×10³) for control cuvettes, 2.6 ± 1.14.
[b] Coronavirus antibody titer, 1:1600 as determined by indirect IFA with TGEV in secondary canine kidney cells as antigen.
[c] Prepared from TGEV-infected A-72 cell tissue culture fluid.
[d] Prepared from mock-infected A-72 cell tissue culture fluid.
[e] ND, Not done.

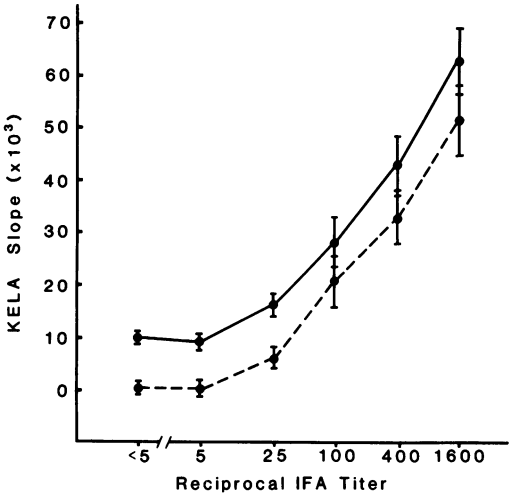

FIG. 1. Comparison (mean) ± (standard error) of uncorrected (——) or corrected (------) KELA slopes and IFA titers (log scale) for 142 individual feline sera with TGEV as the KELA antigen. IFA titers were determined with TGEV in secondary canine kidney cells. The uncorrected KELA slope for a test sample was defined as the mean slope value of several replicates performed with the viral antigen preparation. The corrected KELA slope for a test sample was determined by subtracting the mean slope value of several control antigen replicates from the uncorrected KELA slope, and it represented that portion of the uncorrected slope which could be attributed to coronavirus-specific antibodies. Equivalent results were obtained for 123 additional sera with CCV as the KELA antigen (data not shown).

sera also showed reactivity in empty cuvettes (Fig. 3). However, neither showed an extraordinary relative elevation of reactivity with bovine serum. This suggested nonspecific adherence of serum components to polystyrene-copolymer. Unequivocally coronaviral IFA- and KELA-negative sera showed minimal levels of reactivity with all components, including FBS and NCS, and did not react in the absence of antigen. From this study the following three classes of feline sera were suggested: class 1 sera reacted with bovine serum; class 2 sera reacted uniformly with all components and also reacted in the absence of antigen; and class 3 sera had little or no nonviral reactivity (low-background sera).

Several lots of FBS and NCS were next examined to determine whether this nonviral reactivity was lot specific. In addition, purified goat IgG and GGF-NCS were tested to determine whether this reactivity might be associated with the gamma globulin fraction of serum. All preparations were appropriately diluted in deionized water and dried onto cuvettes as before. Several class 1 feline sera were chosen as probes and tested by using KELA protocol 2. Serum no. 76184 was representative of this class of sera, showing strong reactivity with all preparations except GGF-NCS and control cuvettes (Table 4). These data suggested that some or all of the non-coronaviral reactivity observed in A-72 cell tissue culture fluids resided in the gamma globulin fraction of bovine serum.

To determine whether this reactivity was associated only with bovine (and caprine) serum components or represented a more general phenomenon, sera obtained from several mammalian and avian species were appropriately diluted and dried onto cuvettes as antigens (Table 5). Both class 1 and class 3 sera were chosen as probes, and the various antigen preparations were tested by using KELA protocol 2 (Fig. 4). Reaction of conjugate with the antigen preparations alone (i.e., in the absence of a feline test serum) produced a cross-reactivity profile delineating the background specificities of the conjugate-antigen interaction (Fig. 4A). Uncorrected KELA slopes were greatest with whole cat serum as the antigen, whereas lower reactivity was obtained with human, dog, pig, and guinea pig sera (18, 32, 63). Horse and rat sera showed intermediate levels of reactivity, whereas ruminant, rabbit, chicken, and gamma globulin-free horse serum were least reactive. Similar profiles were produced when cat sera from a minimal-

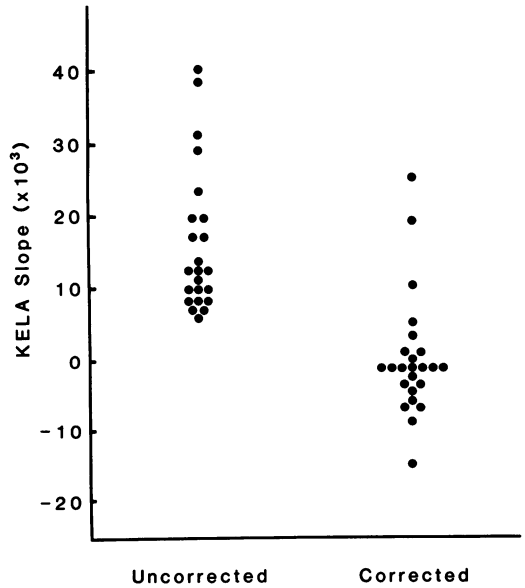

FIG. 2. Scattergram comparing uncorrected and corrected KELA slopes for 23 individual ≤1:25 feline sera, with TGEV as the KELA antigen. IFA titers were determined with TGEV in secondary canine kidney cells. Equivalent results were obtained for 36 additional ≤1:25 sera with CCV as the KELA antigen (data not shown).

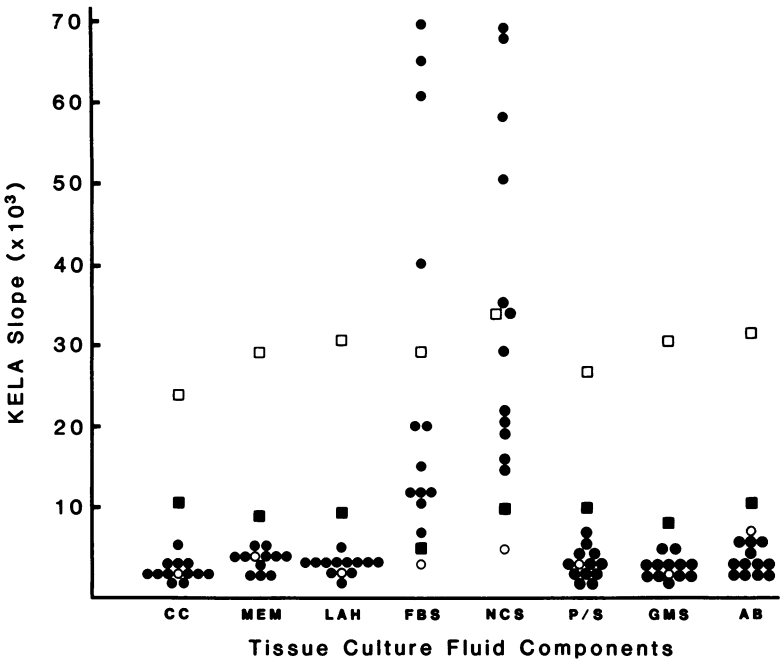

FIG. 3. Determination of A-72 cell tissue culture fluid components responsible for non-coronaviral reactivity. Abbreviations: CC, control cuvette (antigen coating omitted); MEM, Eagle minimum essential medium with Earle salts and L-glutamine; LAH, lactalbumin hydrolysate; FBS, fetal bovine serum (GIBCO lot no. 31P6401); NCS, newborn calf serum (GIBCO lot no. 25K5004); P/S, penicillin-streptomycin; GMS, gentamicin sulfate; AB, amphotericin B. Symbols: ●, class 1 serum samples with representative non-coronaviral reactivity; □ and ■, class 2 sera; ○, low-background control serum representative of class 3.

disease feline breeding colony were tested against these antigen preparations, indicating a lack of cross-reactivity in these sera above the background level of the conjugate (Fig. 4B). Increased reactivity with ruminant sera was observed with many class 1 samples (Fig. 4C–E). Several class 1 (and class 3) sera also showed increased reactivity with rat (Fig. 4C) and (less frequently) chicken (Fig. 4E) sera. Virtually all feline sera tested, regardless of class and coronavirus antibody titer, were minimally reactive with gamma globulin-free horse serum. Taken together, these data suggested that the nonviral reactivity in A-72 cell tissue culture fluid may be a general phenomenon associated with the gamma globulin fraction of sera from a number of animal species.

TABLE 4. Uncorrected KELA slopes for feline serum no. 76184, using bovine and caprine antigen preparations

Antigen prepn	Uncorrected KELA slope ($\times 10^3$)[a]
FBS	
GIBCO lot no. 31P6401	59
GIBCO lot no. 29K5209	54
GIBCO lot no. 31N1102	73
NCS	
GIBCO lot no. 25K5004	71
GIBCO lot no. 29P8001	58
GGF-NCS, GIBCO lot no. C194419	14
Purified goat IgG[b]	130
Control cuvette[c]	11

[a] Determined with KELA protocol 2.
[b] Prepared as described in the text.
[c] Deionized water alone.

Based on the results of the preceding experiments, two modifications of antigen preparation for the KELA were implemented. (i) A-72 cells were adapted to grow in gamma globulin-free serum and cleansed of NCS components by numerous passages in GGF-NCS, accompanied by 48-h fluid changes. The resulting cells grew vigorously and were quite susceptible to infection with either TGEV or CCV. (ii) Viral and control antigen supernatants were diluted in deionized water rather than in carbonate-bicarbonate buffer for preparation of antigen coatings. With these modifications in place, the basic parameters of the KELA were determined (Fig. 5) in preparation for computer-assisted optimization.

TABLE 5. Mammalian and avian sera evaluated for nonviral reactivity in the KELA

Serum	Source
Fetal bovine	GIBCO lot no. 29K5209
Newborn calf	GIBCO lot no. 25K5004
Bobby calf	GIBCO lot no. R693918
Bull	S. Tsai, Cornell University
Goat	GIBCO lot no. A692912
Human	D. F. Holmes, Cornell University
Pig	GIBCO lot no. R198321
Guinea pig	GIBCO lot no. A495412
Rabbit	GIBCO lot no. 25N7001
Dog	GIBCO lot no. A097523
Cat	NYSDL serum no. 89703
Horse	D. F. Holmes, Cornell University
Gamma globulin-free horse	GIBCO lot no. 33K5101
Rat	GIBCO lot no. R797415
Chicken	GIBCO lot no. A096420

Computer-assisted optimization of the KELA. The KELA was optimized by simultaneously testing several dilutions of antigen, serum, and conjugate against one another. The selection of dilution ranges was based on the results of the KELA parameter study (Fig. 5). One negative and one high-positive class 3 serum were chosen for the optimization run. The following dilutions were included in the optimization procedure: antigen, 1:5, 1:10, and 1:20; serum, 1:20, 1:40, 1:80, 1:160, and 1:320; conjugate, 1:1,000, 1:2,500, and 1:4,000. Optimization exploited the high sample throughput potential of the KELA system: multiple replicate samples involving a total of 750 cuvettes were processed over a period of 5 h in a complex "three-dimensional checkerboard" procedure. The separation of uncorrected KELA slopes of negative and high-positive sera was optimal at an antigen dilution of 1:10, a (interpolated) serum dilution of 1:100, and a conjugate dilution of 1:1,000 (data not shown). These parameters were then incorporated into KELA protocol 3 (Table 1). Substrate was still dispensed in 400-μl volumes, however, to minimize occasional refractive effects seen when the light beam of the spectrophotometer passed too near the meniscus of the substrate solution.

Computer-assisted field survey. A total of 181 low-background NYSDL submission sera were evaluated in a computer-assisted field survey using KELA protocol 3 (Fig. 6). Once again, a correlation was obtained between corrected KELA slopes and IFA titers. With this procedure, however, a much more linear correlation with improved titer discrimination at low slopes was achieved (Fig. 6). This was attributed in great part to the use of GGF-NCS in the A-72 cell tissue culture medium, which removed elevated background absorbance and allowed corrected slopes to rise. A nomograph could then be constructed from these data to allow for the direct conversion of corrected KELA slopes to IFA titers, thus preserving a well-established scale of reporting coronavirus antibody titers (manuscript in preparation). Corrected KELA slopes for an additional group of ≤1:25 sera (not shown in Fig. 6) were again found to cluster

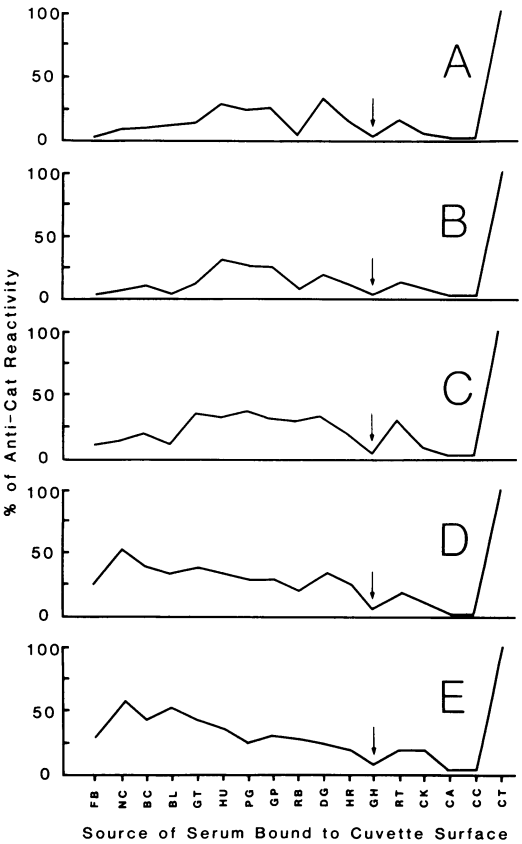

FIG. 4. Cross-reactivity profiles of feline sera with selected mammalian and avian sera as antigens (KELA protocol 2). (A) Conjugate control profile (test serum omitted). (B) Representative class 3 serum from a cat in a minimal-disease feline breeding colony. (C–E) Representative class 1 NYSDL submission sera. Abbreviations: FB, fetal bovine serum; NC, newborn calf serum; BC, bobby calf serum; BL, bull serum; GT, goat serum; HU, human serum; PG, pig serum; GP, guinea pig serum; RB, rabbit serum; DG, dog serum; HR, horse serum; GH, gamma globulin-free horse serum; RT, rat serum; CK, chicken serum; CA, control antigen (deionized water alone); CC, control cuvette (antigen coating omitted); CT, cat serum. Arrows emphasize minimal reactivity consistently observed with GH.

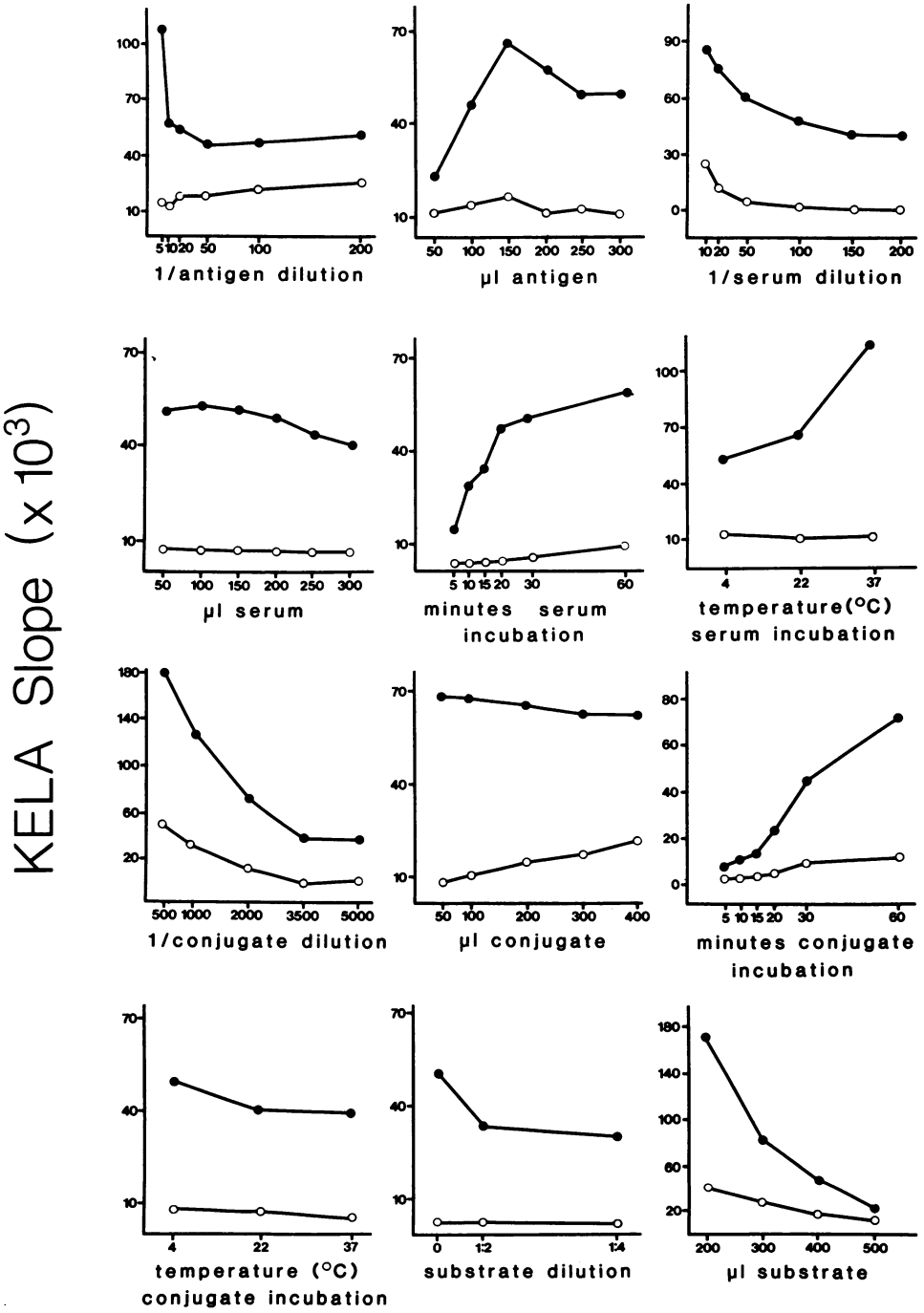

FIG. 5. Effects of different assay parameters on the KELA slope of coronavirus antibody-positive feline sera with viral (●) and control (○) antigen preparations. The basic procedure studied was KELA protocol 2. These data represent mean values for 1, 2, or 3 experiments, each performed in triplicate.

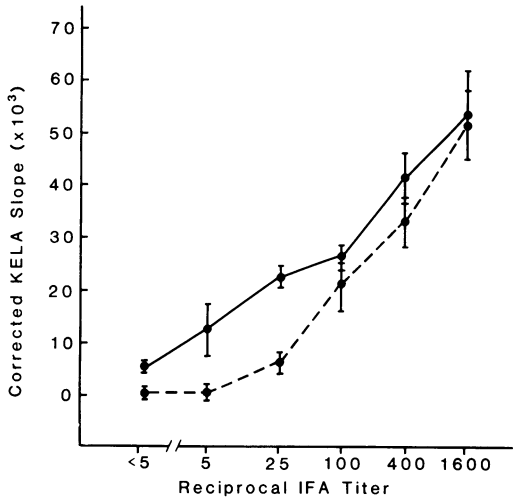

FIG. 6. Comparison (mean) ± (standard error) of corrected KELA slopes and IFA titers (log scale) for 181 individual feline sera, using TGEV grown in A-72 cells in the presence (------) or absence (——) of bovine gamma globulin as the antigen (KELA protocol 3). IFA titers were determined with TGEV in secondary canine kidney cells.

around zero, suggesting that the uncertainty in IFA interpretation of these sera could be attributed to elevated nonviral background reactivity (data not shown).

DISCUSSION

KELA system. In this paper we report the adaptation and evaluation of a computer-assisted KELA system for the detection of coronavirus antibodies in feline serum. Unlike a conventional ELISA, the KELA relies on enzyme-substrate reaction rate kinetics and generates linear quantitative data, eliminating the requirement for serial dilutions of serum. By computer manipulation, these data can then be readily converted to a continuous scale of titers through the use of a well-controlled nomograph (manuscript in preparation). Thus, in our feline system, routine KELA results can be reported as a continuous scale of "IFA-equivalent" titers, circumventing the need for introduction of another scale of measurement (i.e., corrected KELA slopes). By minimizing systematic and random error and maximizing utilization of computer technology, the KELA represents a valuable immunoassay system that is capable of a high sample throughput while providing rapid and accurate kinetics-based detection of analytes. Seroepizootiological studies using this system are currently in progress.

Diffuse color reaction. Adsorption of protein to polystyrene surfaces is a well-established phenomenon and forms the basis of many solid support systems in both ELISA and radioimmunoassay. In ELISA, the classic technique involves passive adsorption of antigen in alkaline diluents such as carbonate, bicarbonate, carbonate-bicarbonate, glycine, or borate buffers (9, 13, 38, 65). Alternatively, antigen may be bound at physiological pH values by using diluents such as PBS (12, 53) or water (55, 72). Fixative and coupling agents for improving antigen retention on support media, such as formaldehyde (12, 25), glutaraldehyde (1, 55, 64), acetone (57), and poly-L-lysine (14, 37) have also been used. Most ELISA protocols for detection of coronavirus antibodies thus far published (including that of Osterhaus et al. [36] for coronavirus antibodies in cats) have used carbonate-bicarbonate or borate buffer diluents at pH 9.2 to 9.8 for antigen adsorption, without additional chemical modification (9, 15, 24, 30, 36, 45, 46, 58). Both infected culture fluids and gradient-purified coronavirus have been used as antigens in these assays.

High nonspecific reactions and nontitratable endpoints associated with the use of unpurified infectious bronchitis coronavirus antigen diluted in carbonate-bicarbonate buffer have been reported (27). Substitution of 0.15 M NaCl as the antigen diluent produced better results which were further improved by the use of gradient-purified virus. In our assay, we also found carbonate-bicarbonate buffer (pH 9.6) to be an inappropriate antigen diluent. After the addition of substrate solution, a visual inspection of cuvettes coated with antigen diluted in this buffer revealed development of a diffuse green color within the substrate solution itself rather than at the cuvette wall surface as expected. Subsequent experiments have shown that this diffuse color reaction could be greatly reduced simply by lowering the pH of the antigen diluent (J. E. Barlough, unpublished data). Optimal reduction was found at pH 5.5 to 7.0. In this range, the diffuse reaction was virtually eliminated and was replaced by the expected development of color on the cuvette walls which gradually diffused into the fluid phase. An evaluation of several other diluents (Table 3) ultimately led to the selection of deionized water (pH 6.3) as the standard diluent for the KELA.

Since the diffuse color reaction could also be easily reproduced by raising the pH of the deionized water diluent from 6.3 to 9.6 (unpublished data), it was not carbonate-bicarbonate buffer dependent. It is possible that the reaction may have been produced by an as yet unidentified pH-dependent antigen present in A-72 cell tissue culture fluids. This antigen may have been bound by feline immunoglobulins in both viral and control antigen cuvettes and could then have been detected by the anti-cat conjugate. If

binding and retention of the antigen to polystyrene-copolymer were reversible at a reduced pH, then rapid elution of the antigen from the cuvette walls upon addition of substrate solution (pH 4.0) could have occurred. This would have rapidly released antigen-antibody-conjugate complexes into the fluid phase and could have produced a diffuse color that would not have been localized on the cuvette walls. Since the diffuse color reaction was not observed when antigen diluents with lower pH values (6.3 to 7.4) were used, it might be that the pH range of 7.4 to 9.6 contains a critical lower limit above which elution is facilitated by the addition of substrate. An alternative hypothesis would be that at alkaline pH, strong initial binding of the antigen to the cuvettes occurred, whereas at a more neutral pH, antigen binding was tenuous. This could partly explain the loss of reactivity observed after prewashing when PBS and water were used as antigen diluents (data not shown). In the absence of prewashing, however, these diluents facilitated binding of specific reagents in subsequent steps, leading to the expected pattern of color development. The question remains as to why the addition of feline test serum and the subsequent PBST wash steps in the remainder of the assay did not dislodge neutral pH-bound antigen (presumably coronavirus), which was apparently susceptible to removal by a PBST prewash. Possibly, binding of immunoglobulins to immobilized coronavirus enhanced its adherence to polystyrene-copolymer.

In addition, we are currently unable to explain the correlation of the intensity and rate of development of the color reaction with IFA titer (Table 2), unless it were postulated that the same antigen was present in the canine kidney cell cultures used for performing the IFA. (It should be noted that the two assays were developed and performed in different laboratories using different media and sera.) Forghani and Schmidt (14), in an ELISA for rubella virus antibodies in human serum, found test sera which reacted with control antigen to almost the same extent as with viral antigen, even after high-speed centrifugation of tissue culture fluids. Sera containing antinuclear antibody showed the highest reactivity with control antigen preparations. Slaght et al. (55) have reported general, nonspecific binding of avian serum to plastic support surfaces, regardless of the presence of viral or control antigen, which in certain cases seemed to be related to the immunoglobulin levels of the test sera. Interestingly, nonspecific binding was found to be maximal at pH 5.5 and could be reduced by precoating wells with FBS. It is conceivable that coronavirus antibody titers in our initial test samples may have roughly paralleled host immunoglobulin levels, in which case nonspecific binding might have given rise to the correlation between coronavirus antibody titer and the intensity of the diffuse color reaction. However, this type of reaction would most likely have been visible initially on the cuvette walls, rather than in the substrate solution itself, and would have been observed also in uncoated cuvettes. Another alternative explanation would be that bovine immunoglobulins present in the antigen preparations might have bound antigens present in feline serum. Clearly, the development of colored product in the fluid phase rather than on the cuvette surface remains unexplained; nevertheless, it is an important observation which must be taken into account as a potential variable in ELISA testing.

Reactivity against heterologous serum components. Another peculiarity of our system which may help to explain variable background activity in individual sera was uncovered during a preliminary field survey which compared KELA results with IFA titers. KELA reactivity against a non-coronaviral component of A-72 cell tissue culture fluids was observed in numerous sera, but was particularly evident in the samples with lower titers, designated $\leq$1:25. Identification of coronavirus-specific immunofluorescence in these samples was hampered by elevated levels of background fluorescence and made precise interpretation difficult. After a series of investigations, much of this non-coronaviral reactivity was shown to be associated with bovine gamma globulins present in the cell culture medium. In almost all cases, reactivity with bovine serum was greater with NCS and adult serum than with FBS, which would be expected since maternal immunoglobulins in bovines are transferred primarily after birth through ingestion of colostrum and milk (7). Conversely, GGF-NCS and gamma globulin-free horse serum were minimally reactive.

It seems likely that this reactivity reflects primarily the presence of antibodies in feline serum samples directed against immunoglobulin (and other) seroantigens from the heterologous species. Tissue culture vaccines prepared for use in cats (as well as vaccines for other animals) contain heterologous serum components (primarily ruminant) which could conceivably induce background antibody reactivity in sera of vaccinees; this reactivity might be detected when sera are tested against tissue culture-derived antigen preparations in assays such as IFA and ELISA/KELA (4, 5, 10, 33, 34, 47). In addition, chlamydial vaccines containing egg-derived organisms contain avian antigens which might be responsible for occasional reactions seen with chicken serum (Fig. 4E) (29). Feline serum or cells, on the other hand, are infrequent components of tissue culture vaccines used in

heterologous species. We have recently obtained a number of feline serum samples drawn within a few days or weeks of host immunization with a variety of vaccine preparations. Many of these sera have been found to contain substantial amounts of background reactivity, and at present there appears to be a general correlation between the presence of this reactivity and the amount of time elapsed since vaccination (J. E. Barlough and C. E. Pepper, unpublished data). This effect has been especially noticeable in sera from young kittens undergoing their primary series of immunizations. Moreover, we have been able to experimentally induce elevated background reactivity in cats by vaccinating them (J. E. Barlough and L. Lesher, unpublished data).

Reactivity against heterologous serum components has been observed in other systems as well. For example, Yolken and Stopa (73), in an ELISA for rotaviral antigen in human feces, found a small number of specimens which reacted non-specifically with normal goat serum. This nonspecific reactivity could be reduced by preincubation with normal goat serum, rabbit anti-human IgM serum, 2-mercaptoethanol, or *N*-acetylcysteine, suggesting the presence of an IgM antibody directed against caprine serum components. Kraaijeveld et al. (24) have presented evidence for the tight adherence of bovine serum components from tissue culture fluid to gradient-purified human coronavirus 229E and mouse hepatitis virus 3. These serum components were highly immunogenic and capable of causing ELISA cross-reactions between the two viruses. Johansson et al. (22) have shown that elevated levels of background fluorescence in IFA tests can be attributed in many cases to antibodies directed against bovine serum components tightly attached to cell surfaces. They speculated that bovine serum components present in tissue culture vaccine preparations might produce antibodies that could participate in this type of reaction. They also indicated that assays using human serum might also be affected by this same type of unwanted fluorescence, and, indeed, others (23) have shown that antibodies against bovine gamma globulins are frequently found in adult human sera.

The implications of these observations for diagnostic assays in which tissue culture-derived antigens are used as probes for antibodies can only be speculated upon. However, it is important to note that, in our assay, the detection of non-coronaviral reactivity (both gamma globulin associated and non-gamma globulin associated) on a routine basis would have been impossible without the inclusion of control antigen cuvettes for each individual sample tested (i.e., generation of corrected slopes). With this refinement of the KELA protocol, it became possible to correct for background reactivity in each sample and thus eliminate a potentially major source of false-positive reactions. Generation of corrected slopes showed that most ≤1:25 sera were actually negative (Fig. 2), and that uncertainty in the interpretation of IFA slides for these sera could be attributed to background reactivity unassociated with coronavirus. In addition, several sera with lower titers which had not been designated ≤1:25 were found to have unexpectedly elevated uncorrected slopes which declined to levels compatible with their IFA titers when corrected slopes were plotted. This suggested that these samples had been properly interpreted by IFA as containing coronavirus-specific reactivity as well as background reactivity. However, the determination of corrected slopes for many sera with higher titers did not improve the correlation. Thus, although the latter samples were unequivocally positive by IFA due to the magnitude of their titers, elevated levels of background reactivity were also present which lowered their corrected slopes without interfering with IFA interpretation. Removal of bovine gamma globulins from the viral and control antigen preparations produced a more linear comparison between slopes and IFA titers, with greater titer discrimination at the lower end (Fig. 6). Although variable levels of non-gamma globulin-associated background reactivity (presumably due to trace gamma globulin reactivity in GGF-NCS and to serum components other than gamma globulins) still exist in some test samples, these modifications minimized their effect and, more importantly, corrected for their presence during slope determination. Analogous procedures to correct for background reactivity in other systems have also been reported (54, 58, 59, 73). However, these assays were conventional ELISAs which relied either on subtraction of control antigen absorbance values to generate specific activity (58, 73) or absorbance difference (59) data or on signal-to-noise ratios of infected to control antigen wells (54). In the KELA, kinetics data are corrected by adjusting enzyme-substrate reaction rate slopes.

Class 2 sera. A few sera (class 2) reacted rather uniformly with all tissue culture components and also reacted in the absence of an antigen coating (Fig. 3). This suggested possible nonspecific adherence of serum components to polystyrene-copolymer (possibly due to immunoglobulin aggregation). Hemolysis of test samples was not associated with this phenomenon. The possibility also existed that catalase-positive bacteria contaminating these samples and surviving the serum and conjugate wash steps might have artificially catalyzed the conversion of substrate to colored product. Bacteri-

al isolation studies of random sera from all three classes did not support this hypothesis, however (J. E. Barlough, unpublished data). Fortunately, class 2 sera have been encountered only rarely and do not seem to represent a significant source of error in data interpretation.

Conclusions. Collectively, these studies indicated that control of nonspecific reactivity in feline coronaviral serology is absolutely essential to obtain useful estimates of specific antibody responses. It is also clear that binding of antigen to polystyrene-copolymer surfaces may require conditions different from those usually applied in ELISA methodology. Even when optimized, antigen binding to cuvettes may be quite variable (unpublished data). Thus, we routinely utilize five replicates of viral antigen and five replicates of control antigen for each sample tested in the NYSDL (manuscript in preparation). Because of computerization, the added replications place no significant additional burden on the technician. With extensive replication it is possible to detect and correct for excessive variation, thus providing a more accurate result.

ACKNOWLEDGMENTS

This work was supported by a grant from the General Committee of the New York State College of Veterinary Medicine, by general research funds from the departments of Microbiology and Preventive Medicine, Cornell University, and by private contributions to the Cornell Feline Health Center.

We acknowledge the expert technical assistance of Leslie Jones, Barbara Lake, Cheryl Stoddart, Giovanna Sorresso, Carol Pepper, Eleanor Tompkins, Peter Sayles, and Cordell Geissinger.

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