The Kinetics-based Enzyme-linked Immunosorbent Assay for Coronavirus Antibodies in Cats: Calibration to the Indirect Immunofluorescence Assay and Computerized Standardization of Results through Normalization to Control Values

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ABSTRACT

The computer-assisted, kinetics-based enzyme-linked immunosorbent assay for coronavirus antibodies in cats was calibrated to the conventional indirect immunofluorescence assay by linear regression analysis and computerized interpolation (generation of "immunofluorescence assay-equivalent" titers). Procedures were developed for normalization and standardization of kinetics-based enzyme-linked immunosorbent assay results through incorporation of five different control sera of predetermined ("expected") titer in daily runs. When used with such sera and with computer assistance, the kinetics-based enzyme-linked immunosorbent assay minimized both within-run and between-run variability while allowing also for efficient data reduction and statistical analysis and reporting of results.

Key words: Enzyme-linked immunosorbent assay, coronaviruses, feline infectious peritonitis virus, transmissible gastroenteritis virus.

RÉSUMÉ

Cette expérience portait sur l'épreuve immunoenzymatique, basée sur la cinétique et l'assistance d'un ordinateur, pour la recherche des anticorps contre le coronavirus félin. Elle visait à la calibrer en fonction de l'épreuve indirecte conventionnelle d'immunofluorescence, par l'analyse de régression linéaire et l'interpolation obtenue par ordinateur, i.e. la génération de titres équivalents à ceux de l'épreuve indirecte conventionnelle d'immunoflorescence. Les auteurs développèrent des procédés de normalisation et de standardisation des résultats de l'épreuve immunoenzymatique précitée, en incorporant dans leurs tests quotidiens cinq échantillons de sérum dont ils connaissaient déjà la teneur en anticorps contre le coronavirus félin. L'utilisation de l'épreuve immunoenzymatique précitée avec les cinq échantillons témoins et l'assistance d'un ordinateur minimisa les variations, tant à l'intérieur d'un test qu'entre plusieurs, tout en permettant de l'efficacité dans la réduction des données, l'analyse statistique et le rapport des résultats.

Mots clés : épreuve immunoenzymatique, coronavirus, virus de la péritonite infectieuse féline, virus de la gastroentérite transmissible.

INTRODUCTION

A computer-assisted, kinetics-based enzyme-linked immunosorbent assay (KELA) for detection of coronavirus antibodies in feline serum has been described (1,2). Unlike conventional enzyme immunoassays, the KELA relies on enzyme-substrate reaction kinetics and generates linear quantitative data (reaction rate slopes), eliminating the requirement for serial dilution of test samples and circumventing error factors associated with the use of stopping reagents and reliance on absolute absorbance values (1,3-7). These data, representing the rate of substrate conversion by peroxidase, can be converted readily to a continuous scale of titers through the use of control sera and a standardized nomograph. Computerized normalization of daily control values to "expected" results and subsequent normalization of individual test sample results to the adjusted control values can then provide standardized data that are directly comparable on a day-to-day basis (4).

In this paper, we describe procedures for (i) conversion of KELA slopes to titers by calibration to the conventional indirect immunofluorescence assay (IFA) for coronavirus antibodies (generation of "IFA-equivalent" titers); (ii) normalization

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and standardization of KELA results through incorporation of control sera of known (expected) titer in daily runs; and (iii) evaluation of within-run and between-run reproducibility of the standardized assay.

MATERIALS AND METHODS

FELINE SERA

All serum samples for coronavirus antibody determinations were submitted to the New York State Diagnostic Laboratory by private veterinary practitioners in the northeastern and eastern United States and by clinicians in the small animal clinic of the Veterinary Medical Teaching Hospital, Cornell University.

KELA

Serum samples were tested for coronavirus antibodies using KELA protocol 3, as described previously (1), with some modifications. Briefly, the Miller strain of porcine transmissible gastroenteritis virus was propagated in canine A-72 cells (8) in growth medium consisting of Leibovitz's L-15 medium (Gibco Laboratories, Grand Island, New York), 10% heat-inactivated gamma globulin-free newborn calf serum (Gibco), 50 μg/mL gentamicin sulfate and 2.5 μg/mL amphotericin B. At 48 to 72 hours after virus adsorption, flasks were subjected to one cycle of freezing and thawing, and the cell culture fluids were centrifuged at 2,000 × g for 20 min at 4°C to remove cellular debris. Supernatant fluids were then pooled and stored in aliquots at -75°C (virus antigen preparation). Control A-72 cells were grown and treated in an identical manner except that virus was omitted (control antigen preparation). To prepare stored cell culture fluid supernatants for the KELA procedure, a sample of each of the two preparations was quickly thawed and diluted 1:10 in cold (4°C) sterile de-ionized water (pH 6.3 to 6.5), and dispensed in 200 μL quantities in 750 μL polystyrene-copolymer EIA cuvettes (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Individual Gilford reaction vessels consisted of ten such cuvettes, each having a 1 cm wavelength path, fused together into a single strip. The first five cuvettes of each strip were coated with the virus antigen preparation and the last five cuvettes with the control antigen preparation. Coated strips were then dried at 37°C in an unhumidified incubator for 36 to 40 hours and stored at 4°C for no more than a week prior to use. Testing for coronavirus antibodies was performed using the semi-automated Gilford EIA System 50 under direct computer control (1,4). One serum sample was tested per strip, so that five replicate slope values for each of the two antigen preparations was obtained for each sample. The *uncorrected KELA slope* for a test sample was defined as the mean slope value of the five replicates performed with the virus antigen preparation. The *corrected KELA slope* for a test sample was determined by subtracting the mean slope value of the five control antigen replicates from the uncorrected KELA slope; it thus represented that portion of the uncorrected KELA slope that could be attributed to coronavirus-specific antibody.

The titer of each test sample was determined via normalization to five control sera, which served as internal standards. Each control serum represented a pool of sera of similar titer derived from individual cats. The titers of these five controls were established by running them at least ten times in either a nonnormalized (initial five controls) or normalized (replacement controls) KELA over a period of weeks in order to obtain overall mean corrected KELA slopes for each. By nomographic interpolation (Fig. 1), each control serum was then assigned an "expected" IFA-equivalent titer, a value that was preserved as a standard for that control throughout all subsequent KELA runs. The five titer levels of the control sera, ranging from negative (serum A) to approximately 1:1000 (serum E), were selected in order to provide a spread that was representative of the general range of IFA titers seen in clinical submissions to the New York State Diagnostic Laboratory (9). The reproducibility of the KELA was examined by studying within-run and between-run variabilities for the five control sera.

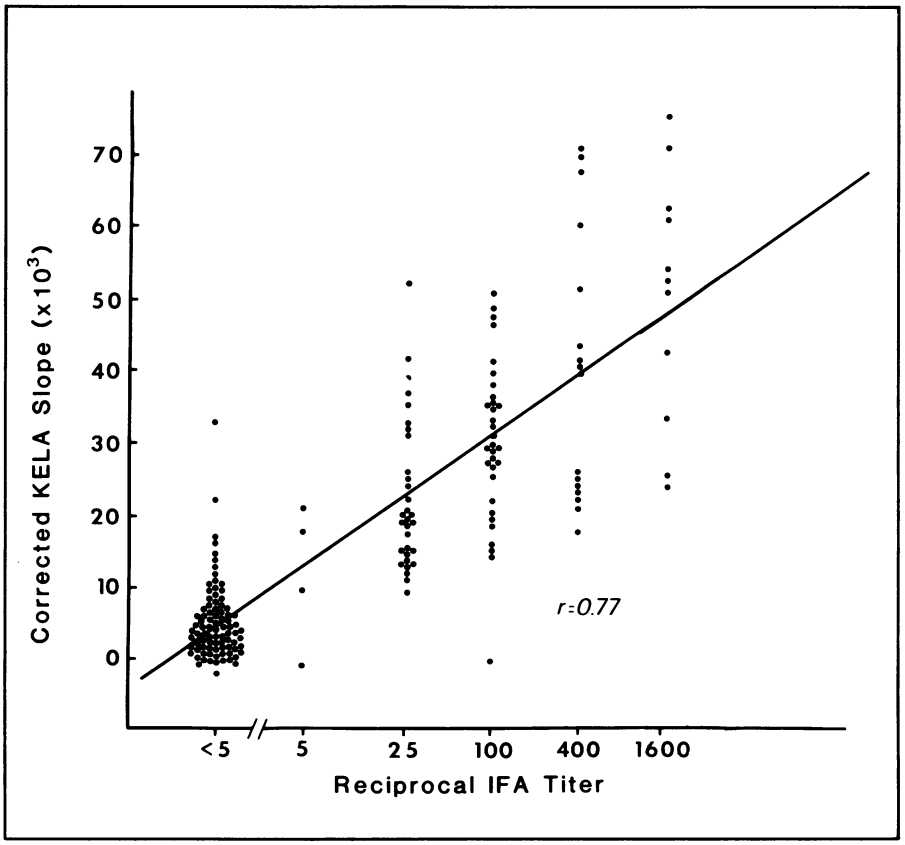

Fig. 1. Regression of corrected KELA slopes on IFA titers for 181 individual feline serum samples. The data were rectified by plotting titers on a $\log_{10}$ scale. Individual slopes could be converted to "IFA-equivalent" titers by computerized interpolation.

INDIRECT IFA

The Miller strain of transmissible gastroenteritis virus was grown in either secondary canine kidney cells or in A-72 cells, and coronavirus antibody assays performed using either glass microscope slides or disposable plastic Leighton tubes (Costar, Cambridge, Massachusetts), as described previously (9). Test sera were diluted 1:5, 1:25, 1:100, 1:400, and 1:1600. Samples without specific viral fluorescence at a dilution of 1:5 were considered negative. A group of sera was also identified that showed elevated levels of background fluorescence at low to moderate dilutions, making positive identification of virus-specific fluorescence difficult. Sera showing such elevated background fluorescence were designated "≤ 1:25" and were excluded from consideration in this study for reasons that have been delineated elsewhere (2).

RESULTS

CONVERSION OF KELA SLOPES TO IFA-EQUIVALENT TITERS

In order to define the relationship between corrected KELA slopes and indirect IFA titers, a total of 181 feline sera were evaluated by both techniques. Following computer-assisted linear regression analysis (Fig. 1), KELA slopes were converted to a continuous scale of IFA titers and individual values (including those of the five normalizing control sera) were determined by computerized interpolation (10,11). In this way, the need for introduction of a new scale of measurement (corrected KELA slopes) was circumvented, and KELA results were reported to veterinarians in terms with which they were familiar (IFA titers). Technically, of course, these results represented IFA-*equivalent* titers, since they were generated by an enzyme immunoassay and converted to titers by nomographic comparison.

COMPUTERIZED NORMALIZATION AND STANDARDIZATION OF KELA RESULTS

By including the five control sera of known (expected) titer in each subsequent run, daily KELA results were standardized via computerized normalization of test sample data. As a first step in the daily normalization procedure, the corrected KELA slopes (y-axis) obtained that day for the five control sera were run in linear regression analysis against the $\log_{10}$ of their expected titers (x-axis) in order to establish the nomograph (Fig. 2). Once fitted, the five control sera provided a correlation coefficient (r) which typically approached 1.000. The titer for any given corrected slope was then determined from the nomograph. Computer-generated statistical analyses provided information (mean, standard deviation, coefficient of variation) on the replicates of each control and test sample, and on the linearity of the daily regression line (r).

As expected, reaction rates of the enzyme-substrate interaction were found to vary from day to day due to differences in assay conditions, such as changes in ambient temperature or in reagents used (4,5). Accordingly, corrected slope values varied somewhat between runs. This variability, however, affected all control and test samples proportionally; i.e. the degree of variation was proportional to slope value (and hence to antibody titer). Thus the linearity of the slope/titer relationship was preserved (Fig. 2). Although a given test serum replicated on several days had slightly different slope values each day, a constant calculated titer was obtained. Titers thus were determined independent of varying assay conditions, allowing direct comparison of results from day to day.

REPRODUCIBILITY OF THE KELA

The reproducibility of the standardized KELA was well within acceptable limits (Table I). The normalization procedure was of great value in reduction of within-run and between-run variability (decreased standard deviations and coefficients of variation). As expected, however, coefficients of variation were artificially greater in samples of extremely low titer (negatives).

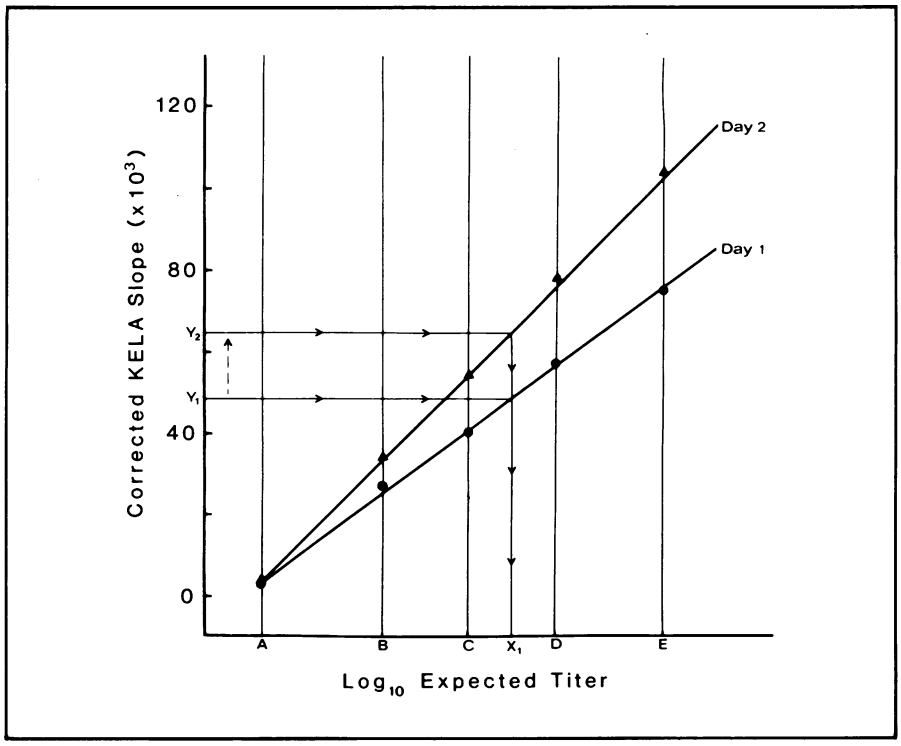

Fig. 2. Daily standarization of KELA results through computerized normalization to control sera. *Day 1*: A regression line was fitted to the comparison, corrected KELA slope vs. $\log_{10}$ expected titer, for the five control sera (A through E). The corrected KELA slope (Y_1) of a given test serum was then converted to a calculated $\log_{10}$ (IFA-equivalent) titer (X_1). *Day 2*: Elevated corrected slopes resulting from a change in assay conditions (new lot of anti-cat conjugate) have increased the pitch of the regression line. However, the new corrected slope values for the five control sera have increased in proportion to the expected titers, thus preserving the linearity of the slope/titer relationship. The overall effect is to accommodate for variation in assay conditions while maintaining constant titer levels. Thus the $\log_{10}$ of the antibody titer (X_1) of the given test serum remained unchanged on day 2 even though the absolute value of its corrected slope had increased (arrow) from Y_1 to Y_2. (Data taken from Table I).

TABLE I. Within-run and Between-run Reproducibility of the KELA for Coronavirus Antibodies[a]

Within-run						
	Corrected KELA Slope ($\times 10^3$)					
Test Sera	Nonnormalized			Normalized		
(No. of Replicates)	Arithmetic Mean	SD[b]	%C.V.[c]	Arithmetic Mean	SD	%C.V.
A(8)	2.6	1.06	40.4	2.7	0.88	32.2
B(8)	36.2	2.96	8.2	26.0	1.85	7.1
C(8)	56.1	2.10	3.7	40.1	1.36	3.4
D(8)	79.8	5.41	6.8	55.7	3.49	6.2
E(8)	105.1	5.14	4.9	74.4	3.42	4.6
Between-run						
	Corrected KELA Slope ($\times 10^3$)					
Test Sera	Nonnormalized			Normalized		
(No. of Replicates)	Arithmetic Mean	SD	%C.V.	Arithmetic Mean	SD	%C.V.
A(8)	2.4	1.06	44.2	2.7	1.80	66.8
B(8)	28.9	5.59	19.3	28.0	3.23	11.5
C(8)	39.1	6.58	16.8	37.9	3.26	8.6
D(8)	60.5	3.16	5.2	61.2	1.17	1.9
E(8)	68.4	2.62	3.8	70.6	1.82	2.6

[a]Representative experiment. Nonnormalized slope values differed between within-run and between-run data because of a change in the KELA conjugate used. Normalization preserved the slopes (and hence the titers) of these five samples (see Fig. 2)

[b]SD, Standard deviation

[c]C.V., Coefficient of variation

DISCUSSION

In an earlier paper (1), we described the adaptation of the computer-assisted KELA to detection of coronavirus antibodies in feline serum. In this system, enzyme-substrate reaction kinetics provide linear quantitative data on a continuous scale that are directly proportional to antibody titers. The KELA system was chosen for its inherent objectivity and its theoretical insensitivity, via normalization, to time, temperature, and reagent variations encountered in most day-to-day operations of manual or noncomputer-assisted, semi-automated enzyme immunoassays (4). In the present report, we have described methods for conversion of corrected KELA slope data to IFA-equivalent titers, and for normalization and standardization of results through the use of control sera of predetermined titer. When used with such sera and with computer assistance, the KELA minimizes virtually all sources of variation while allowing for efficient reduction, normalization, and reporting of data (1,4).

Systematic and random assay error, coupled with variability in quality of immunological reagents, has limited the usefulness of conventional immunoassay systems for many years and precluded meaningful standardization of results. The KELA system thus may serve as a model of the level of technology that may be required if serotest standardization between laboratories is ever to become a reality. Theoretically, calculation of expected titers for a series of control sera in one laboratory with an established KELA system should provide the basis for standardization of the test in a second laboratory which subsequently employs the controls in its own KELA. Later, replacement controls can be prepared by first determining their expected titers and then substituting them in the assays as the original control pools are depleted. Minor differences in reagents used in the second laboratory are accommodated for with the normalization procedure. Establishment of a standardized serotest such as the KELA should be especially welcomed by feline coronavirus serologists, who inhabit a murky world characterized at best by a plethora of test methodologies and the chaotic absence of any recognizable attempt at standardization of results (or of their interpretation) between laboratories (12).

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