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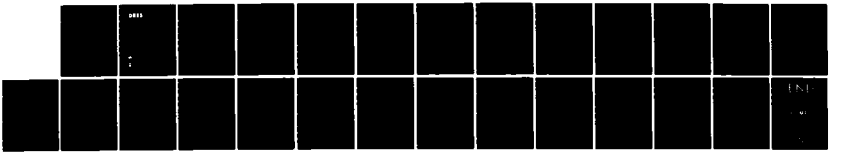

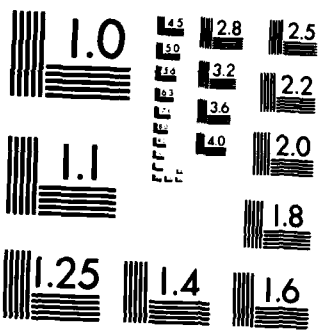

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RAPID RATE-KINETIC TURBIDOMETRIC ASSAY FOR QUANTITATION OF ANTIVIRAL COMPLEMENT-FIXING ANTIBODIES (U)

by

R.E. Fulton and V.L. DiNinno

Project No. 16B10

February 1985

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PREFACE

Complement fixation tests are used routinely and universally in clinical diagnostic laboratories for the identification and quantitation of specific antibodies. The technique may also be applied to the identification of infectious agents. Conventional complement fixation tests are complicated and time-consuming to perform, sometimes requiring up to three days to complete. Results are difficult to interpret and unbiased readings are not easily achieved. The rate-kinetic turbidometric assay described in this paper offers an alternative technique for the performance of complement fixation tests; it is simple, sensitive and accurate, and the assay time required is short. Instrumentation for the test

PREFACE (Continued)

allows for the simultaneous assay of six different reactions. There is, however, commercially available instrumentation which can easily be adapted to the assay and which would allow for the simultaneous monitoring of up to 352 reactions.

The rate-kinetic turbidometric assay greatly facilitates performance of complement fixation tests and brings their useage into the realm of rapid identification techniques. The method may be applied to automated and rapid identification of infectious agents (in aerosols or in patients' specimens) and/or specific agent antibodies (in patients' sera) and hence is pertinent to the interests and responsibilities of Defence microbiologists.

This work was presented, in part, in poster format, at the Sixth International Congress of Virology, Sendai, Japan, 1 – 7 September 1984.

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ABSTRACT

A rapid rate-kinetic turbidometric assay for the quantitation of viral complement fixing antibodies has been developed, using adenovirus as a model. The procedure is based on the turbidometric quantitation of intact sheep erythrocytes and measures the rate of hemolysis (change in absorbance per min at 640 nm) at maximum velocity occurring in the presence of residual complement not fixed by the antigen-antibody reaction. Reagents were standardized and assays performed using a microprocessor-controlled spectrophotometer with thermoregulated cell compartment and kinetic assay capability. Eleven sera were assayed for complement fixing antibodies both by the

ABSTRACT (Continued)

conventional microtiter technique and by the rapid turbidometric method described here. Good correlation (r = 0.88) between the two procedures was obtained. Unlike the conventional complement fixation test, in the rate-kinetic turbidometric complement fixation test, it was found that variation in complement and antigen concentration could be tolerated, end-point titers were objectively quantitated, and results could be obtained within one hour. The technique may be adaptable to large-scale automation,

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INTRODUCTION

Spectrophotometric methods, based on the turbidometric quantitation of erythrocytes during the complement activation process, have made it possible to design highly sensitive and reproducible systems for complement titration. In spite of the merits afforded by spectrophotometric methods, this approach has not been widely exploited in the diagnosis of clinical disease. In this paper a turbidometric assay for the quantitation of complement fixing (CF) antibodies to adenovirus has been described. The technique involved incubating antigen and specific antibody in the presence of complement and then assessing the amount of complement fixed by measuring the rate of loss in absorbance of indicator erythrocytes. Turbidity was measured at frequent intervals over a relatively short reaction time, thus permitting rapid rate-kinetic analysis of results. The technique combines the features of speed, sensitivity, accuracy, and reproducibility and may be adaptable to automated analysis of multiple samples.

METHODS AND RESULTS

Turbidometric Assay Procedures

A Beckman DU-8 microprocessor-controlled recording spectrophotometer with thermoregulated cell compartment, automatic cuvette advance, and kinetic assay capability was used for experimentation and data acquisition. Six samples were monitored at a temperature of 37°C and a wavelength of 640 nm, continuously at 15 sec – 1 min intervals, throughout a 15 – 30 min reaction period. Reaction mixtures were prepared using veronal buffered saline (VBS) as diluent and were held on ice prior to assay. Appropriate controls were included in each assay.

The rate of hemolysis, defined as the change per min in absorbance at 640 nm (ΔA_{640}/min) was obtained directly from the data output of the spectrophotometer. Maximum rates of hemolysis, i.e., ΔA_{640}/min at maximum velocity (V_{max}) (ΔA_{640}/min/V_{max}) were calculated from this information.

Standardization of Reagents

A. Erythrocytes

Sheep erythrocytes (SRBC) were washed in VBS and the concentration standardized experimentally by constructing a standard curve relating SRBC concentration and A_{640} nm. A suspension of SRBC with A_{640} in the range of 0 to 1.5 nm was on the linear portion of this standard curve. Hence, a working solution of SRBC consisted of a suspension which, when added to the final reactants, produced an A_{640} of approximately 1.0. SRBC at a concentration of 0.1% satisfied this criterion.

B. Titration of Hemolysin

Hemolysin (Flow Laboratories) was titrated in the presence of SRBC and excess complement (lowest dilution of complement not causing spontaneous lysis of SRBC). Serial two-fold 1 mL volume dilutions of hemolysin were prepared in VBS. To 50 μL aliquots of each hemolysin dilution was added 100 μL complement diluted 1/10 (excess), 50 μL VBS, and 400 μL of 0.1% SRBC. The mixtures were stored on ice, then transferred to cuvettes and assayed turbidometrically at 37°C. Maximum rates of hemolysis were determined for each dilution of hemolysin and a curve relating hemolysin

dilution and $\Delta A_{640}/min/V_{max}$ was constructed (Fig. 1). The optimal dilution of hemolysin, defined as the highest dilution required to cause the maximum rate of hemolysis at V_{max}, was determined from this curve to be 1/40.

C. Titration of Complement

Guinea pig complement (Behring Diagnostics) was titrated in the presence of SRBC and the optimal dilution of hemolysin. Serial two-fold 1 mL volume dilutions of complement were prepared in VBS. To 100 μL aliquots of each complement dilution was added 50 μL of hemolysin diluted 1/40 (optimum), 50 μL VBS, and 400 μL of 0.1% SRBC. The mixtures were stored on ice, then transferred to cuvettes and assayed turbidometrically at 37°C. Maximum rates of hemolysis were determined for each dilution of complement and a curve relating complement dilution and $\Delta A_{640}/min/V_{max}$ was constructed (Fig. 2). From this curve, the optimal dilution of complement, defined as the dilution corresponding to 50% of the maximum rate of hemolysis, was determined to be 1/50.

From a comparison of the complement titration plotted as a function of rate of hemolysis and percent hemolysis, $(100 - [\frac{A_{640} \text{ at } 30 \text{ min}}{A_{640} \text{ at } 0 \text{ min}} \times 100])$, it could be seen that the rate curve was less steep in the central portion and thus could be useful over a greater range of complement activity than could the percent hemolysis curve (Fig. 3).

D. Titration of Antigen

Adenovirus CF antigen (Microbiological Associates) was titrated, utilizing a checkerboard type assay technique, against a pool of standard reference human antisera. Serial two-fold dilutions of antigen and of reference antiserum (titer 1/16 by conventional CF test) were prepared in 1 mL volumes. To 25 μL of each dilution of antigen was added 25 μL of each respective dilution of antiserum and 100 μL of complement diluted 1/50 (optimum). Tubes were then placed in an incubator at 37°C and complement fixation was allowed to proceed for 45 min. At the end of this reaction period, tubes were transferred to an ice bath and to each was added 50 μL of hemolysin diluted 1/40 (optimum) and 400 μL of 0.1% SRBC. Reaction mixtures were transferred to cuvettes and assayed turbidometrically at 37°C.

Maximum rates of hemolysis were determined for each dilution of antigen in combination with each dilution of reference antiserum and each was expressed as a percentage of the maximum rate of hemolysis of the complement control (Table 1).

The optimal dilution of antigen was determined to be 1/32, resulting in an antiserum titer of 1/16. However, if antigen dilutions of 1/8, 1/16, or 1/64, respectively, had been selected as optimum, antiserum titers obtained would not have differed significantly (Fig. 4). The technique, therefore, was found to be tolerant of variations in antigen concentration.

Clinical Evaluation

Eleven samples of human sera from patients with known recent infection with adenovirus were assayed both by the rate-kinetic turbidometric assay and by the conventional microtiter CF test. For the turbidometric assay, two-fold 1 mL volume dilutions of antisera were prepared and a 25 μL aliquot of each incubated at 37°C for 45 min with 25 μL of the optimal dilution (1/32) of antigen and 100 μL of the optimal dilution (1/50) of complement. Reaction mixtures were transferred to ice and to each was added 50 μL of the optimal dilution (1/40) of hemolysin and 400 μL of 0.1% SRBC. Reaction mixtures were then transferred to cuvettes and assayed turbidometrically at 37°C. Maximum rates of hemolysis ($\Delta A_{640}/min/V_{max}$) were determined for each dilution of antiserum and each was expressed as a percentage of the maximum rate of hemolysis of the complement control (% $\Delta A_{640}/min/V_{max}$). Log dilutions of each serum assayed were then plotted as a function of % $\Delta A_{640}/min/V_{max}$ and the titer of each antiserum, defined as that dilution causing 50% of the maximum rate of hemolysis (50% $\Delta A_{640}/min/V_{max}$), was read directly from the graphs (Figs. 5 and 6).

The titer of each antiserum determined by rate-kinetic turbidometric assay was compared with that obtained by conventional microtiter CF test (Table 2). A highly significant correlation coefficient ($r = 0.83$) was obtained.

DISCUSSION AND SUMMARY

A rapid rate-kinetic turbidometric assay for the quantitation of viral CF antibodies was developed, using adenovirus as a model. The technique offered the

following features:

1. Assay time was short. Results were obtained in 15 to 30 min, thus interference from decay of labile components was minimized.

2. Multiple data points were obtained for each serum dilution, thus increasing the reliability of determinations.

3. Hemoglobin did not interfere with absorbance of intact cells at 640 nm, therefore serum samples containing hemoglobin could be assayed by this technique.

4. Presensitization of SRBC was not necessary.

5. The technique was tolerant of variations from optimum in concentrations of complement and antigen.

6. The technique has combined the features of simplicity, and sensitivity; the element of subjective interpretation has been removed, thus allowing greater accuracy and reproducibility.

7. The assay may be easily automated for rapid analysis of multiple samples, with computer processing of results. The MS-2 Microbiology System (Abbott Laboratories), with a capability of up to 352 simultaneous assays, is particularly suited to this purpose.

Figure 1

Titration of Hemolysin. Hemolysin was titrated in the presence of SRBC and excess complement. Maximum rates of hemolysis were determined for each hemolysin dilution and the optimal dilution of hemolysin (1/40) identified. Each data point represents the mean of at least six separate determinations. Error bars, representing the standard error of the mean, are indicated.

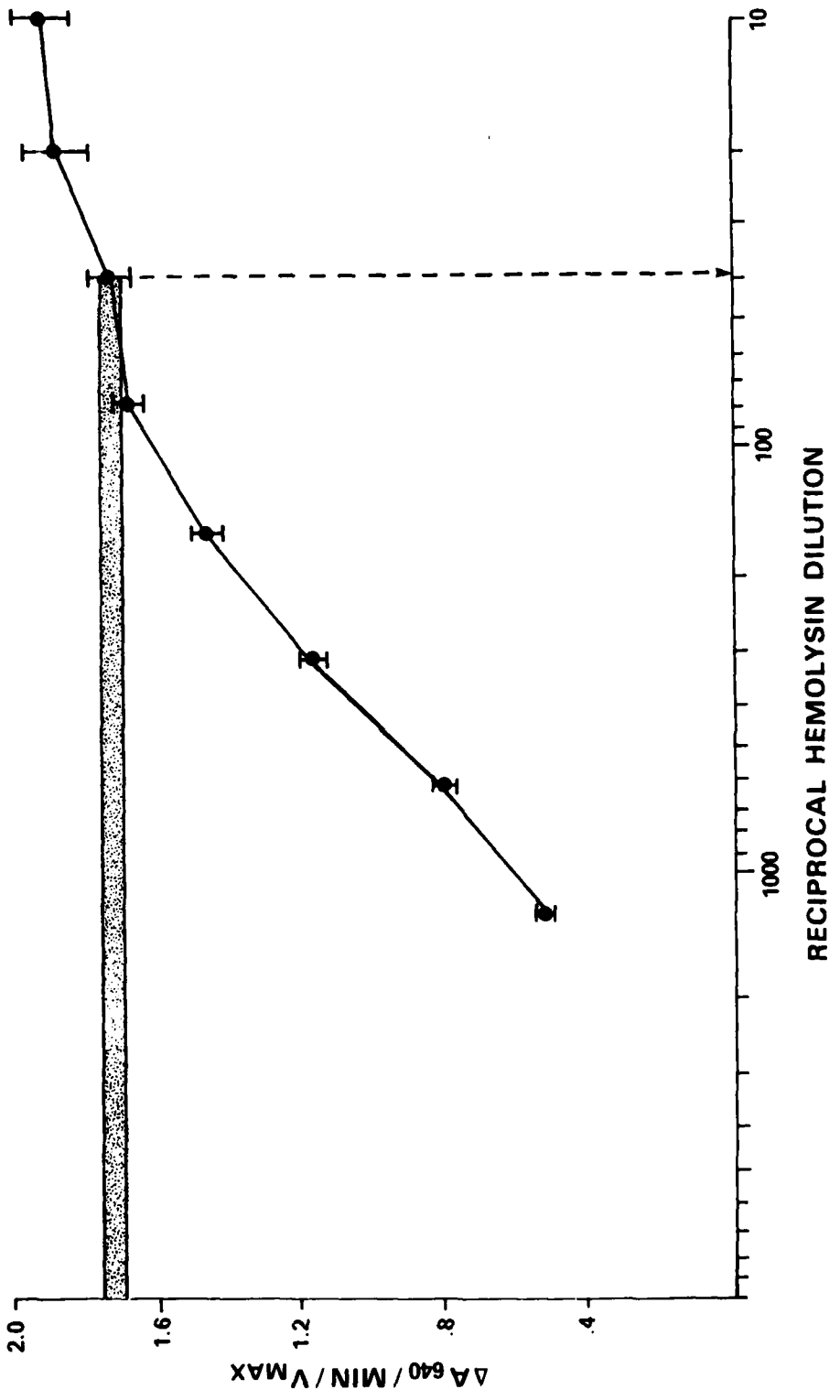
85-12
10
100
1000
RECIPROCAL HEMOLYSIN DILUTION
2.0
1.6
1.2
.8
.4
ΔA 640 / MIN / V MAX

Figure 1

Figure 2

Titration of Complement. Complement was titrated in the presence of SRBC and the optimal dilution of hemolysin. Maximum rates of hemolysis were determined for each complement dilution and the optimal dilution of complement (1/50) identified. Each data point represents the mean of six separate determinations. Error bars, representing the standard error of the mean, are indicated.

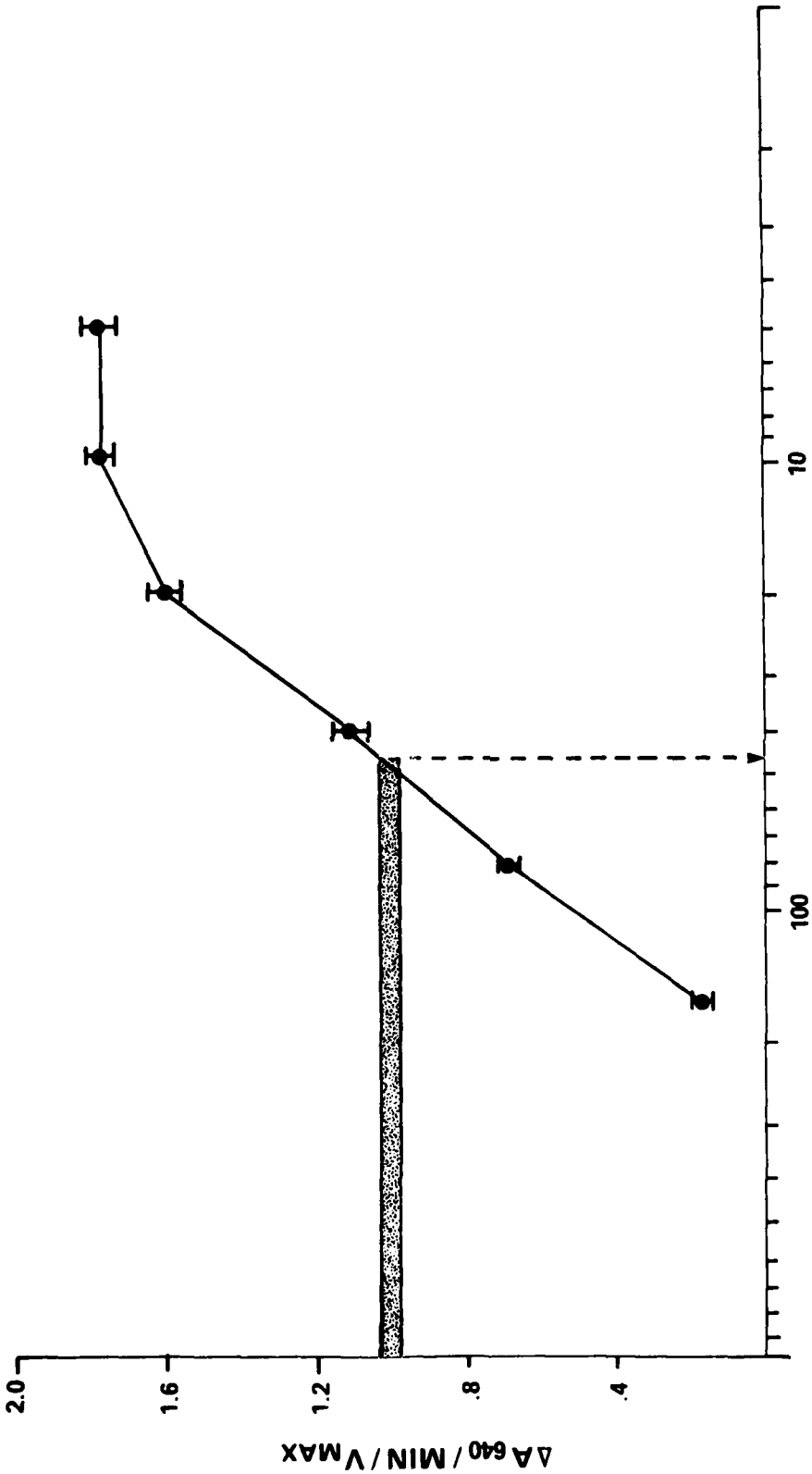
2.0
1.6
1.2
.8
.4
ΔA640 / MIN / VMAX
10
100
RECIPROCAL COMPLEMENT DILUTION

Figure 2

Figure 3

Comparison of Complement Titration Plotted as a Function of Rate of Hemolysis and Percent Hemolysis. The rate curve in its central region is less steep than the percent hemolysis curve, indicating that the rate curve is useful over a wider range of complement concentration. O—O, percent (%) hemolysis; ●—●, rate of hemolysis ($\Delta A_{640}/min/V_{max}$). Error bars, representing the standard error of six separate determinations, are indicated.

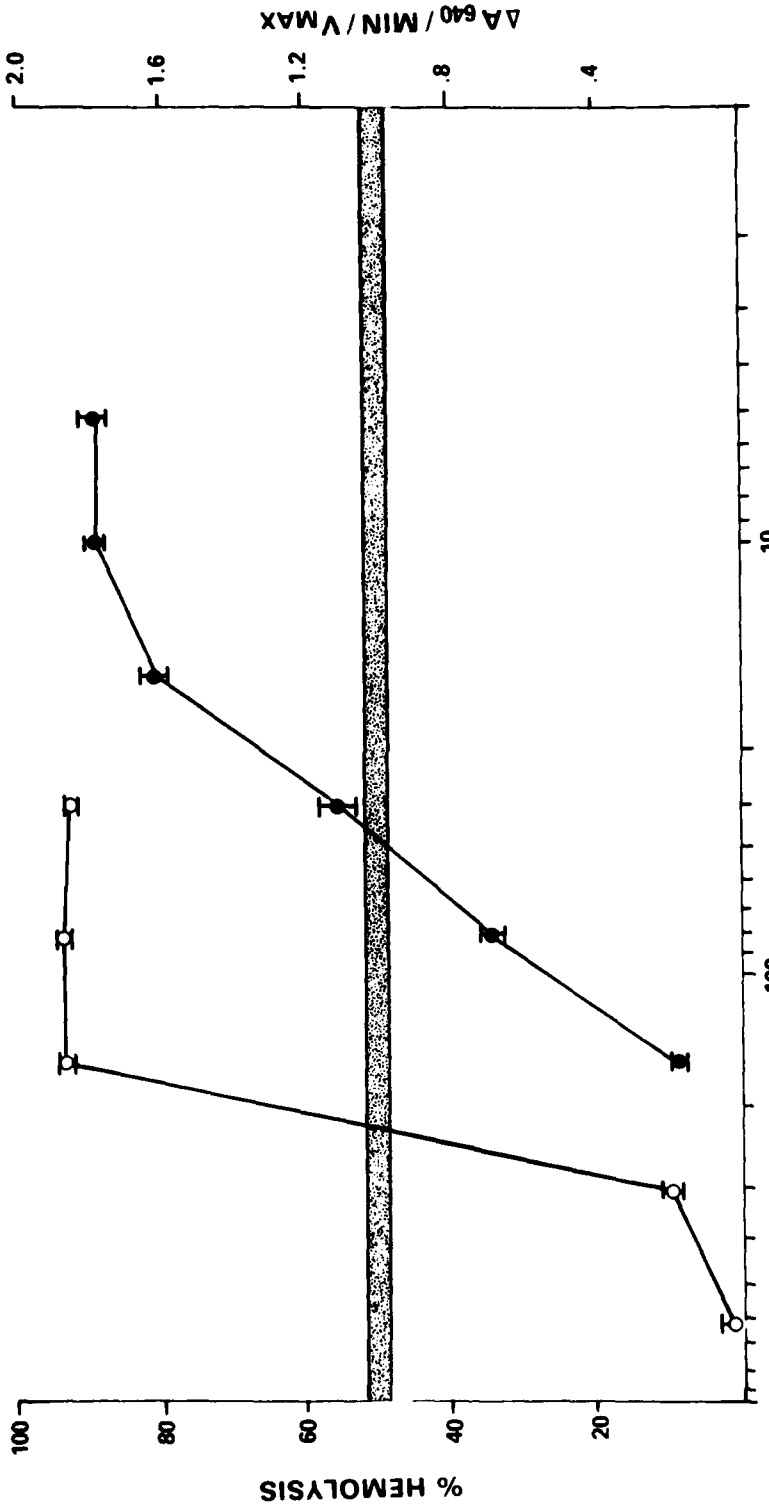
ΔA640 / MIN / VMAX
2.0
1.6
1.2
.8
.4
% HEMOLYSIS
100
80
60
40
20
10
100
RECIPROCAL COMPLEMENT DILUTION

Figure 3

Figure 4

Effect of Optimal Dilution of Antigen on Titer of Antiserum. Selection of the optimal dilution of antigen at 1/8 (●—●), 1/16 (○—○), 1/32 (■—■), or 1/64 (▲--▲) resulted in antiserum titers of 1/9, 1/12, 1/16 and 1/18, respectively, thus indicating that variations in antigen concentration could be tolerated. Error bars represent the standard error of the mean of six separate determinations.

85-12

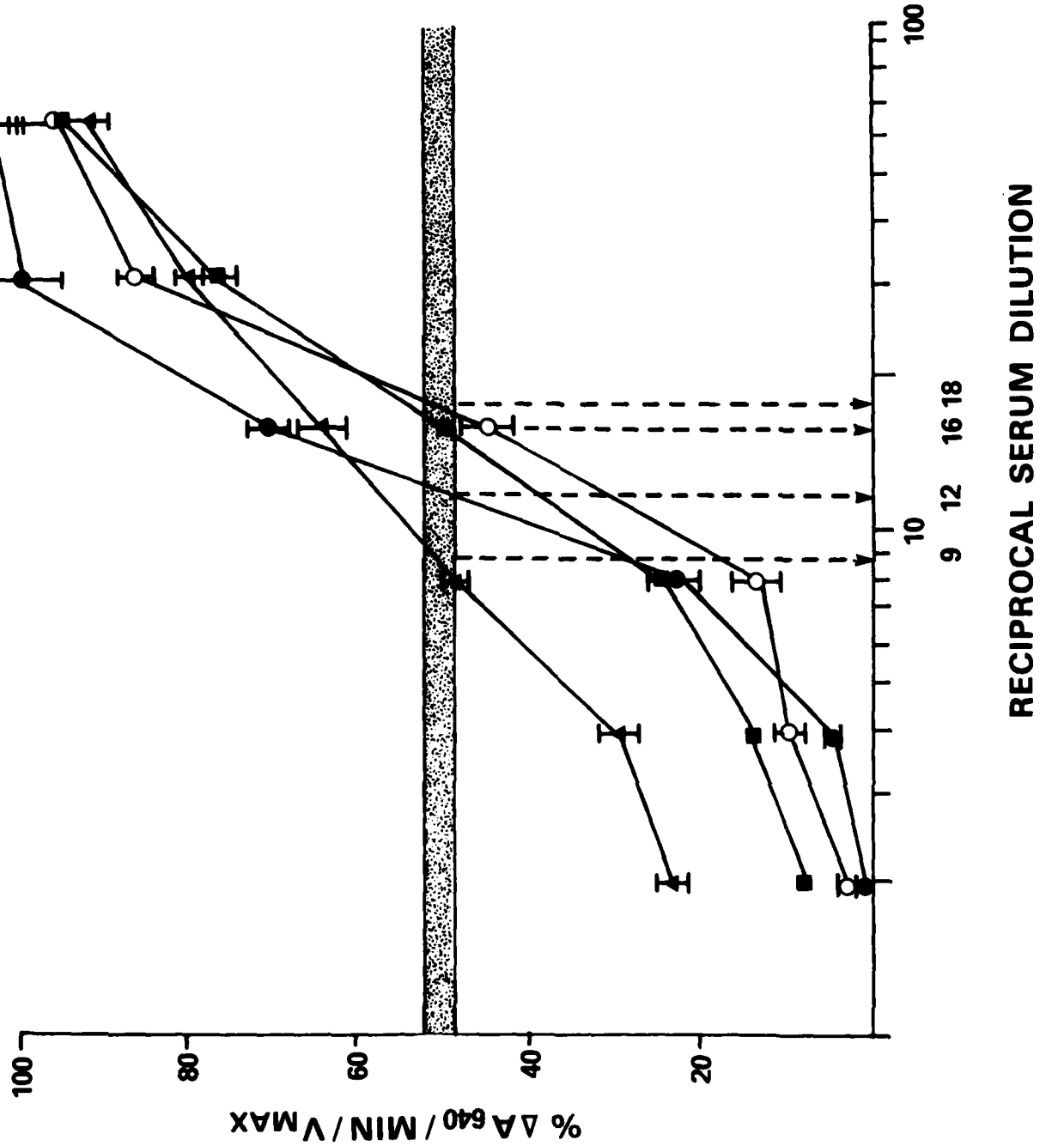

Figure 4

Figure 5

Titration of Antisera. Single serum samples from three patients with known recent infection with adenovirus were titrated by rate-kinetic turbidometric assay. The titer of each, defined as the dilution causing 50% $\Delta A_{640}/min/V_{max}$, was read directly from the graphs.

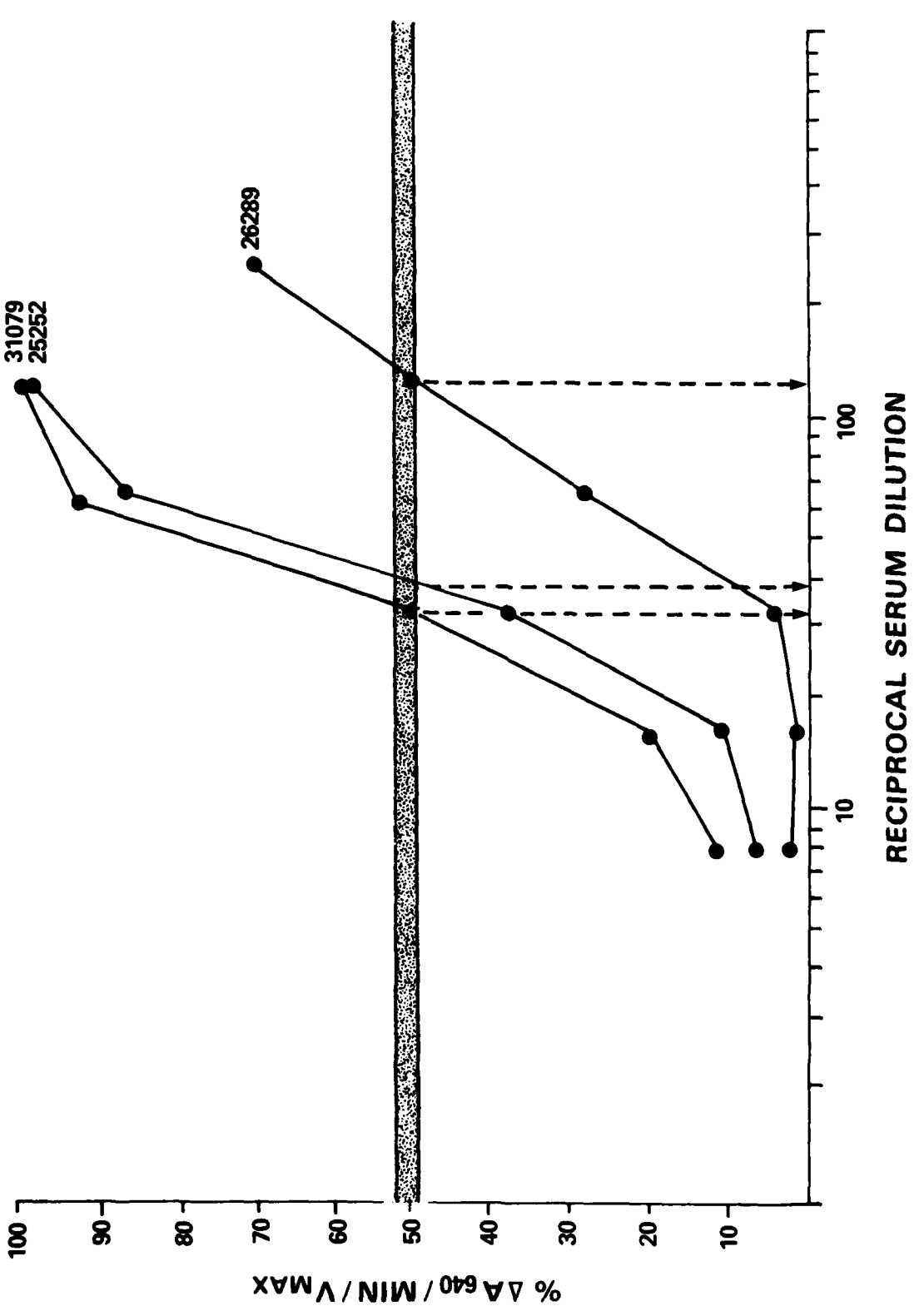

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Figure 5

Figure 6

Titration of Antisera. Acute and convalescent phase serum pairs from four patients with known recent infection with adenovirus were titrated by rate-kinetic turbidometric assay. The titer of each, defined as the dilution causing 50% $\Delta A_{640}/\text{min}/V_{max}$, was read directly from the graphs.

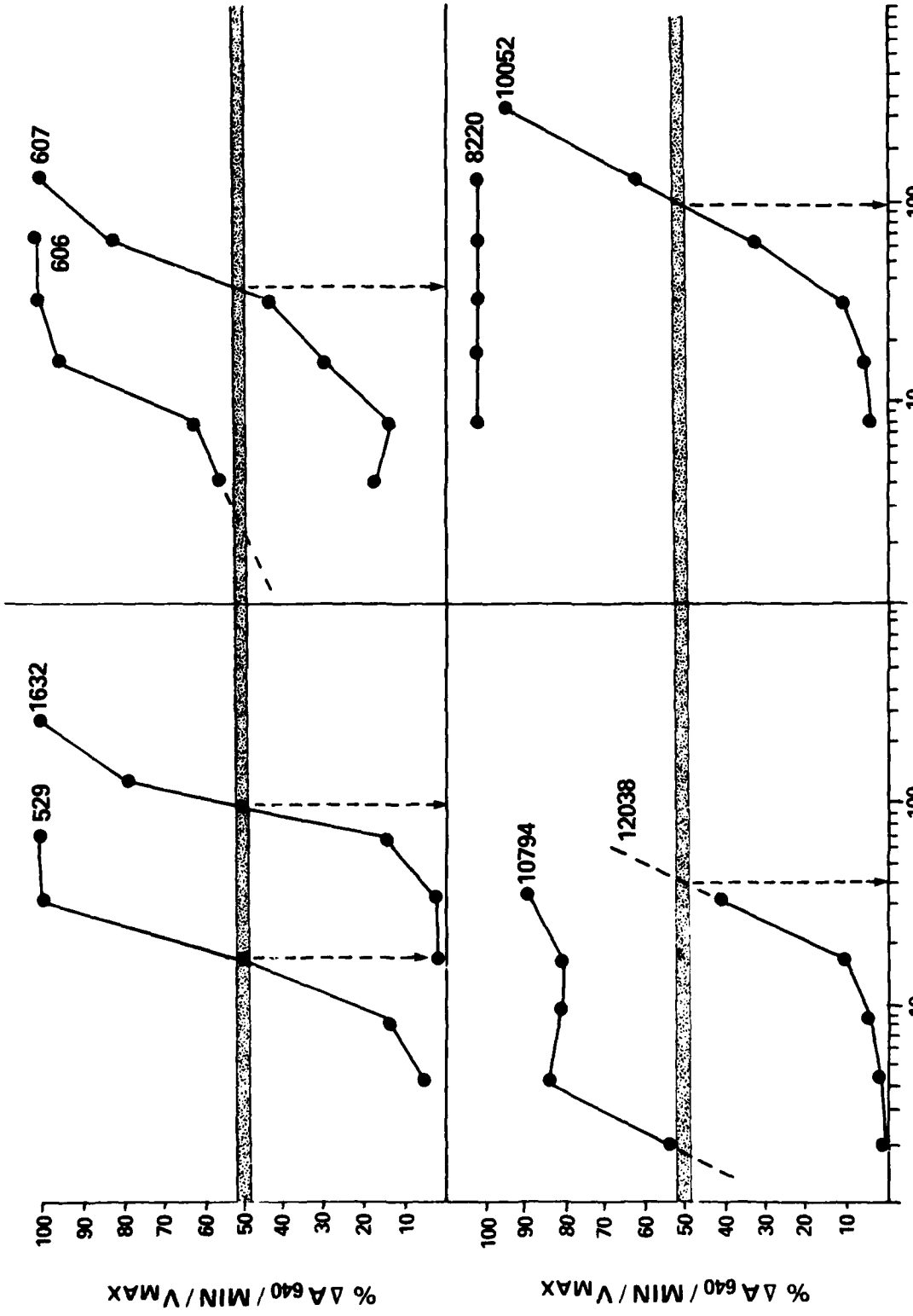
607
606
1632
529
8220
10052
10794
12038
100
90
80
70
60
50
40
30
20
10
% ΔA640 / MIN / VMAX
RECIPROCAL SERUM DILUTION
10
100

Figure 6

ΔA_{640}/min/Vmax

(% ΔA_{640}/min/Vmax)

ANTISERUM DILUTION	ANTIGEN DILUTION 2	4	8	16	32	64	SC
2	.05 ± .01 (5.0 ± .7)	0 (0)	0 (0)	.02 ± .00 (2.0 ± .0)	.07 ± .00 (7.2 ± .4)	.21 ± .00 (23.3 ± 1.9)	.80 ± .02 (89.1 ± 2.5)
4	.30 ± .02 (33.2 ± 1.0)	1.0 ± .01 (10.5 ± 1.0)	.06 ± .00 (6.6 ± .3)	.08 ± .01 (8.6 ± 1.0)	.12 ± .00 (13.0 ± .4)	.26 ± .03 (28.9 ± 3.4)	.87 ± .00 (97.1 ± .4)
8	.66 ± .02 (73.7 ± 1.9)	.41 ± .03 (45.3 ± 3.6)	.20 ± .02 (22.0 ± 2.4)	.12 ± .03 (13.2 ± 3.0)	.22 ± .01 (24.4 ± 1.2)	.43 ± .01 (48.2 ± .7)	.92 ± .03 (101.7 ± 3.6)
16	.90 ± .03 (99.8 ± 3.4)	.91 ± .01 (101.0 ± 1.0)	.63 ± .03 (69.9 ± 3.3)	.40 ± .04 (44.9 ± 4.1)	**.45 ± .01 (49.7 ± 1.0)**	.58 ± .04 (64.3 ± 4.1)	.89 ± .04 (98.3 ± 4.4)
32	.95 ± .03 (105.6 ± 3.2)	.92 ± .02 (102.0 ± 2.1)	.90 ± .05 (99.8 ± 5.2)	.77 ± .02 (85.5 ± 2.3)	.70 ± .03 (77.2 ± 3.4)	.72 ± .01 (79.4 ± 1.4)	.90 ± .02 (99.8 ± 2.0)
64	.87 ± .07 (96.8 ± 8.2)	.86 ± .03 (95.4 ± 3.0)	.92 ± .04 (102.7 ± 4.3)	.85 ± .03 (94.7 ± 3.6)	.85 ± .06 (94.6 ± 6.1)	.83 ± .02 (91.9 ± 2.7)	.92 ± .01 (102.0 ± 1.1)
AC	.85 ± .02 (94.9 ± 2.4)	.89 ± .04 (98.8 ± 4.6)	.98 ± .04 (109.2 ± 4.5)	.91 ± .05 (101.6 ± 5.2)	.95 ± .08 (105.0 ± 8.8)	.93 ± .07 (103.0 ± 7.7)	

Table 1. TITRATION OF ANTIGEN. Maximum rates of hemolysis (ΔA_{640}/min/Vmax) were determined for each antigen dilution in combination with each dilution of reference antiserum. Values represent the mean and standard error of at least three replicate experiments. Maximum rates of hemolysis, expressed as a percentage of the mean maximum rate of hemolysis of the complement control (% ΔA_{640}/min/Vmax) are indicated in brackets. The optimal dilution of antigen (1/32), defined as the highest dilution required to cause 50% of the maximum rate of hemolysis (50% ΔA_{640}/min/Vmax) with the highest dilution of antibody, was determined from the checkerboard titration.

SERUM NO.	TURBIDOMETRIC ASSAY	CONVENTIONAL CF TEST
31079	32	32
25252	36	64
26289	128	256
529	16	16
1632	94	64
8220	< 2	8
10052	100	32
10794	< 2	2
12038	40	32
606	< 2	8
607	40	32

Table 2. COMPARISON OF TITERS BY RATE-KINETIC TURBIDOMETRIC ASSAY AND CONVENTIONAL CF TEST. Brackets indicate acute and convalescent phase sera from the same subject. Correlation coefficient (r) = 0.88.

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