Rubella Neutralizing Antibody Determinations with the Rabbit Kidney Cell Strain LLC-RK_1 (33389)

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Accurate rubella antibody determinations are of importance for the documentation of maternal and congenital infection, as well as for the evaluation of the efficacy of gamma globulin, drugs, and vaccines. The recent development of a hemagglutination-inhibition (HI) test for detection of rubella antibody has provided a method that appears to be rapid and reliable. With this method, however, variation may occur due to inhibitors and other factors in the test. To document HI antibody findings, duplicate determinations as well as an alternate assay procedure should be used to confirm results.

The present report describes our findings for the serum neutralization test using the continuous line rabbit kidney cell strain LLC-RK_1 (1) and compares these results with those obtained with the HI test for rubella antibody.

Materials and Methods. Tissue culture. Strain LLC-RK_1 cell cultures were produced by Microbiological Associates, Bethesda, Maryland. Cells were grown in screw-cap roller tubes with minimum essential medium (MEM) and Hanks' balanced salt solution, containing 10% fetal calf serum, 25 μg chlortetracycline/ml, and 2 m*M* glutamine/ml. The maintenance medium consisted of MEM with Earle's balanced salt solution, 3% fetal calf serum, 25 μg chlortetracycline/ml, 1.0% glutamine, and .01% $NaHCO_3$, adjusted to a final pH of 7.4.

Virus assay. The Gilchrist strain of rubella virus passed 15 times in primary African green monkey kidney and 6 times in RK_1 was employed in these studies (2). Assays for infectivity were performed by inoculation of 0.2-ml quantities of half-log dilutions into each of three tissue culture tubes per dilution. A simultaneous virus titration, with known negative serum (1:4) as a diluent instead of Hanks' solution, was also performed in the same manner. Cultures were incubated in a stationary position at 32°. End points were determined by the presence or absence of cytopathic effect (CPE), after five to seven days' incubation, and were calculated by the method of Reed and Muench (3).

Serum neutralization tests. Screening tests were performed using 1:4 dilution of serum. The dilutions (0.4 ml) of inactivated serum (56°, 30 min) were mixed with equal volumes of the RK_1-adapted rubella virus containing 30–100 $TCID_{50}$ and were incubated for one hr at room temperature. The mixtures were inoculated (0.2 ml) into each of three tissue culture tubes per serum dilution. Each serum had a control for toxicity that consisted of equal volumes of serum with Hanks' solution. The cultures were incubated in a stationary position at 32° with no media change for the duration of the test. Results were recorded on the fifth to seventh day of the test as 1+ to 4+ CPE. End points were recorded as the highest serum dilution showing a 3+ CPE; 4+ CPE was considered negative for rubella antibody. All specimens were tested twice under code.

Hemagglutination-inhibition test. Hemagglutination antigen (HA) was prepared according to the methods reported by Halonen *et al.* (4). The antigen had a titer of 32 units. HA and HI tests were performed as previously described, using the microtechnique similar to the one described by Sever *et al.* (5). For the HI test, .025-ml serial dilutions of sera were made with spiral loops in dextrose-gelatin-barbital diluent. Four units of HA antigen were added (.025 ml) and the mixture was incubated at room temperature for one hr. A suspension of one-day-old chicken red blood cells (0.16%) in dextrose-

TABLE I. Reproducibility of Duplicate Titers of Single Sera from Pregnant Women Using LLC-RK_1 Cell Line.

	Test I		
	Positive	Negative	Total
Test II			
Positive	35	2	37
Negative	2	22	24
Total	37	24	61

gelatin-barbital diluent was added in a volume of .025 ml and the red cells were allowed to settle at 4° overnight. Antibody titer was reported as the highest dilution of serum causing complete inhibition of agglutination. All specimens were tested twice under code. Initially the lowest dilution tested was 1:8; however, later all sera showing no HI antibody at 1:8 were retested at 1:4.

Serum specimens. Serum samples were obtained from pregnant women who participated in the Collaborative Perinatal Research investigation. All sera were coded and randomized before testing.

Results. To determine reproducibility of the RK_1-neutralization tests, duplicate assays of acute and convalescent sera were performed on different days with fresh dilutions of sera in separate lots of tissue (Table I). In the first test, of a total of 61 sera, 37 showed the presence of antibody and 24 were negative. In the second test, 2 of the original positive sera were negative and 2 of the negative sera of the first test were positive. Based on this data, the reproducibility for positive test was 95% and for negative tests 92%.

To determine the specificity and sensitivity of the RK_1 neutralization test, paired sera from 13 patients with clinical rubella were tested and results compared with the HI antibody test using the same sera. All 13 seroconversions were detected with both methods.

The sensitivity of the RK_1 neutralization test for detecting antibody in adult pregnant women was also compared to the HI method. Both tests were run twice under code and individually reproduced (Table II). The presence of rubella antibody in both the neutralization and the HI test was shown in 37 of the sera and not detected in either test in 28 sera. Thus, for positive tests there was 97% correlation. For the negative tests there was 72% agreement. Eleven sera that were positive in the neutralization tests were negative in the HI, and one serum that was negative in neutralization was positive in the HI. In both cases, the latter serological findings were reproduced by the respective methods under code.

In an effort to clarify the finding of neutralizing activity in the absence of HI titer, the 39 sera showing no HI antibody were retested in duplicate at a 1:4 dilution. At this lower dilution, two of the eleven sera that were negative for HI antibody and positive in the neutralization test became positive in HI, three sera had a trace reading at 1:4 dilution and four sera were negative at 1:4. At the lower dilution then, there were two additional HI-negative sera that were positive; however, there remained nine sera that would have to be considered positive with the neutralization test and negative in the HI test (24%).

Discussion. The use of serological techniques for the diagnosis of rubella or susceptibility to rubella carries with it important clinical responsibilities. Most laboratories are currently employing the HI test for this purpose. In our laboratory all HI tests are reproduced under code and all negative plus 10% of the positive tests are rerun using the LLC-RK_1 neutralization method.

We have previously reported that variations in the performance of the rubella HI test can result in false negative or false positive tests (Sever *et al.*) (5). Factors of pri-

TABLE II. Comparison of Antibody Determination with LLC-RK_1 Neutralization Test and Hemagglutination-Inhibition Test on Single Sera.

	Hemagglutination inhibition		
	Positive (≥8)	Negative (<8)	Total
LLC-RK_1			
Positive (≥4)	37	11	48
Negative (<4)	1	28	29
Total	38	39	77

mary importance include method and duration of treatment of sera, type of antigen employed, and age of the red blood cells. All titers should be reproduced under code and comparative tests should be performed with alternate methods. Furthermore, the results of the present study demonstrate the importance of conducting the test at the 1:4 dilution to identify as many positives as possible.

The RK_1 neutralization test is particularly useful for confirming HI results since distinctive and quantitative differences in inhibition of CPE can be detected without difficulty. Reproducibility of the neutralization test was found to be quite good (93%). The sensitivity and specificity of the neutralization test was clearly shown by the assay of paired serum samples of patients with clinical rubella. These results are in agreement with previously reported studies of Hull *et al.* utilizing this cell line (1).

The failure to detect HI antibody for nine sera in which neutralizing antibody was present is of particular importance. Both tests were reproduced under code. It is obviously necessary to determine which of these observations correlate with susceptibility. These opposing results occurred with 24% of the HI antibody-negative tests and must be resolved before clinical interpretations can be made.

These differences in results emphasize the need for comparative testing. Some of the factors that may have contributed to the results include: (1) low levels of antibodies in some serum specimens (better correlation was obtained between the two tests when the HI test was run at a 1:4 dilution); (2) excessive kaolin adsorption in the HI test, possibly removing low levels of antibody; and (3) inadequate removal of nonspecific inhibitors of agglutination or loss of neutralizing activity of the serum. To further identify the validity of the results, tests including the above considerations are being pursued.

Summary. The serum neutralization test utilizing the continuous line of rabbit kidney cell strain LLC-RK_1 has been found to be quite sensitive and reproducible. Some differences in results were found when neutralization tests were compared to HI tests. Performance of the HI test at an initial 1:4 dilution of serum appeared to be an important step in decreasing the possibility of false negative results. Even with this change, of 37 sera with no detectable HI antibody, 9 had antibody in the neutralization test. Clearly, there is a need to not only document serological results but to determine the true significance of these conflicting findings. These differences are important for the interpretation of serological tests for all clinical and vaccine studies.

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