

Comparative Measurement of Equine Influenza Virus Antibodies in Horse Sera by Single Radial Hemolysis, Neutralization, and Hemagglutination Inhibition Tests

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Single radial hemolysis (SRH), neutralization (NT), and hemagglutination inhibition (HI) tests were carried out on sera from horses immunized against the Prague and Miami strains of equine influenza virus. The HI and NT tests demonstrated good sensitivity; the sensitivity of the SRH test was somewhat lower. The NT titers of individual sera were correlated very closely with the HI titers, although the NT titers were higher. SRH zone diameters of individual sera also showed significant correlation with the NT and HI titers. The SRH test appears to be suitable for large-scale serological surveys and offers the advantages of rapidity and simplicity.

For serological evaluations of influenza virus antibodies in horse sera after infection or vaccination, the hemagglutination inhibition (HI) test has been used widely. The HI test has the advantage of good sensitivity, but great care is necessary to achieve reproducible results; one disadvantage is that sera must be treated to remove nonspecific inhibitors and hemagglutinins before the sera can be tested. Recently, we developed a neutralization (NT) test for the Miami and Prague strains of equine influenza virus, in which microplate cultures of ESK cells derived from a swine embryo kidney are used (11). This test is simple enough for routine use and is almost as sensitive as the HI test. The single radial hemolysis (SRH) test has been used to measure influenza virus antibodies in human sera (3, 6, 7, 9), swine sera (5), and horse sera (1). This test is rapid and simple and has a sensitivity similar to that of the HI test. It requires only small amounts of serum and is not affected by nonspecific inhibitors.

In this study we compared results of HI, NT, and SRH tests with sera from horses immunized against the Miami and Prague strains of equine influenza virus.

MATERIALS AND METHODS

Viruses. A/equine/Prague/1/56 ($H_{eq1}N_{eq1}$) (8) and A/equine/Miami/1/63 ($H_{eq2}N_{eq2}$) (10) were used. These strains were grown in the allantoic cavities of 10-day-old chicken embryos. Allantoic fluid was harvested after incubation at 34°C for 48 h, clarified by centrifugation, and stored at -80°C until use. The infectivities of the preparations were $10^{7.0}$ and $10^{9.0}$ 50% egg

infective doses per ml for the Prague and Miami strains, respectively.

Horse sera. Initially, seronegative horses were immunized with hemagglutinin vaccine (4) prepared with the Miami or Prague strain. Each horse was inoculated subcutaneously with two 1-ml doses of the vaccine at intervals of 4 weeks. Serum samples were obtained 4 weeks after the last dose. Sera were stored at -20°C until use.

HI test. The serum for the HI test was inactivated at 56°C for 30 min and treated with receptor-destroying enzyme to remove nonspecific inhibitors and with packed chicken erythrocytes to remove antibodies to the erythrocytes. The HI test was performed by the microtiter method (2), and the HI antibody titer was expressed as the reciprocal of the highest serum dilution showing complete HI with 4 hemagglutinin units. HI titers of 1:8 or higher were taken as positive. In each test run a negative control serum and a positive control serum were included.

NT test. The NT test was performed with microplate cultures of ESK cells derived from a swine embryo kidney by using the method developed in our laboratory (11). ESK cells were grown in Eagle minimal essential medium containing 10% tryptose phosphate broth and 10% calf serum at 37°C in atmosphere of 5% CO₂ in air for 4 or 5 days. In the wells of a transfer plate 0.025-ml portions of serial twofold dilutions (in Hanks solution containing 0.11% bovine serum albumin) or serum inactivated at 56°C for 30 min were mixed with 0.025-ml portions of Hanks solution containing 100 50% tissue culture infective doses of virus. Four wells were used per serum dilution. The virus-serum mixtures were incubated at 37°C for 2 h, transferred into wells of microplates containing ESK cell monolayers, and incubated in a CO₂ incubator at 34°C for 7 days. The antibody titer was expressed as the reciprocal of the serum dilution showing a 50% end-point of neutralization, which was calculated by the

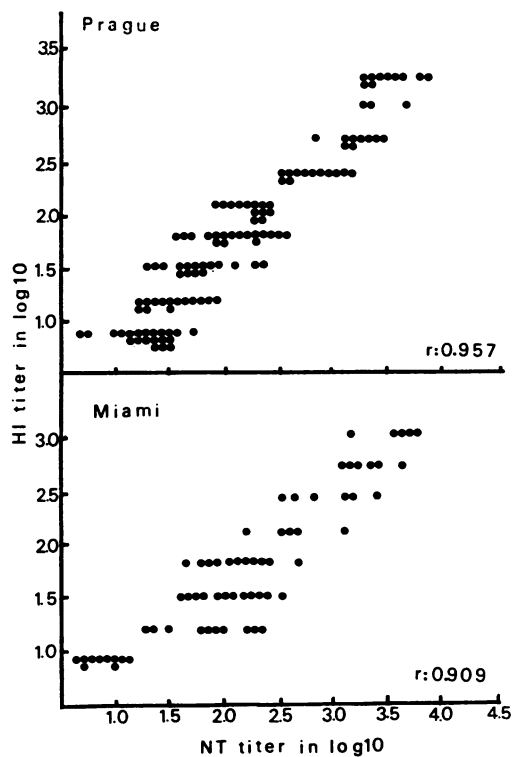


FIG. 1. Correlation between HI and NT antibody titers of individual horses immunized against the Miami and Prague strains.

method of Kärber. Titers of 1:4 or higher were taken as positive. In each test run a negative control serum and a positive control serum were included.

SRH test. One volume of 10% washed chicken erythrocytes in phosphate-buffered saline (pH 7.4) was mixed with 1 volume of infected allantoic fluid containing 1,024 hemagglutinin units per 0.05 ml, and this preparation was incubated at 4°C for 10 min to allow the virus to adsorb to the erythrocytes. The cells were washed by two cycles of low-speed centrifugation and resuspended in 1 volume of phosphate-buffered saline. The resuspended erythrocytes were then incorporated in 1.0% agarose containing 0.1% sodium azide by mixing 0.3 ml of 10% sensitized erythrocytes and 0.1 ml of fresh guinea pig complement with 2.6 ml of molten agarose. The mixture was poured into plastic immunoplates (78 by 28 mm) and allowed to harden. Wells (diameter, 3 mm) were punched in the agarose, and 5 μ l of undiluted test serum inactivated at 56°C for 30 min was added to each well. The plates were incubated at 4°C for 18 h and then at 37°C for 3 h. The diameters of the hemolysis zones were measured, and diameters of 5 mm or more were taken as positive. In each test run a negative control serum and a positive control serum were included.

RESULTS AND DISCUSSION

Two groups of serum samples were tested. The Prague group consisted of 115 samples from

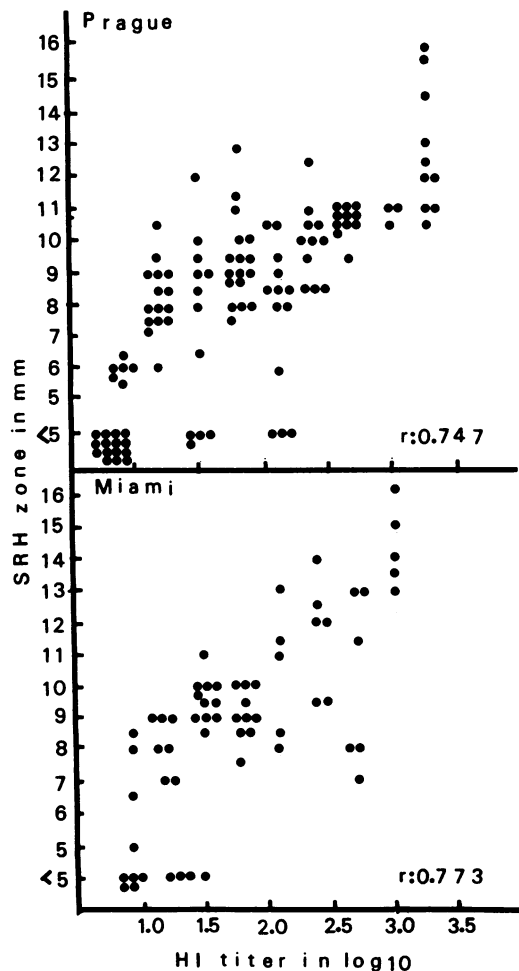


FIG. 2. Correlation between SRH zone diameter and HI antibody titer of individual horses immunized against the Miami and Prague strains.

horses immunized against the Prague strain, and the Miami group consisted of 65 samples from horses immunized against the Miami strain. HI, NT, and SRH tests on the sera of the Prague and Miami groups were carried out by using the Prague and Miami strains, respectively. The HI and NT tests demonstrated good sensitivity, whereas the SRH test was somewhat lower in sensitivity than the HI and NT tests. Thus, all of the serum samples of both groups were positive in the NT and HI tests, whereas the SRH tests were positive for 93 of 115 sera (80.9%) of the Prague group and for 56 of 65 (86.2%) of the Miami group. Previously, we reported that the NT test is as sensitive as the HI test (Yamagishi et al., in press).

For a further comparison of these tests, correlations between the HI titer, the NT titer, and the SRH zone diameter of individual serum

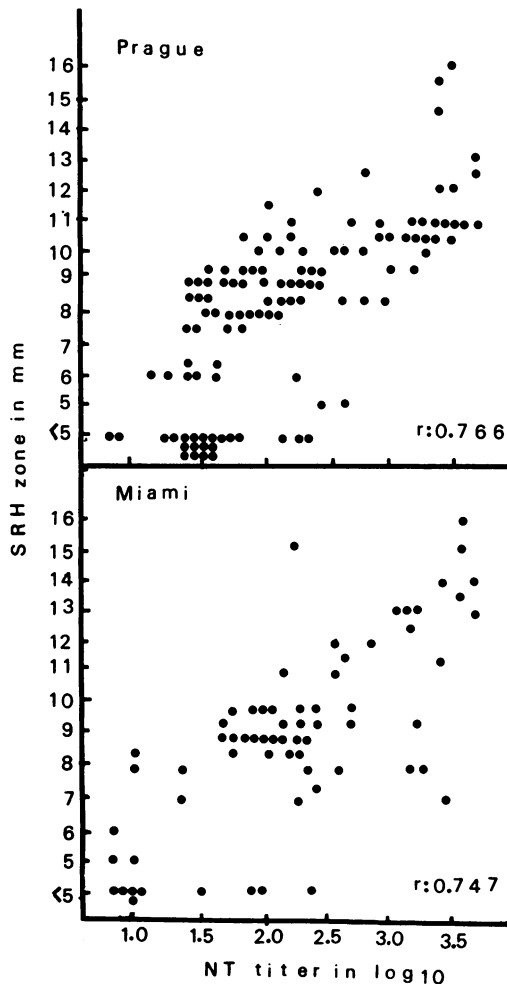


FIG. 3. Correlation between SRH zone diameter and NT antibody titer of individual horses immunized against the Miami and Prague strains.

specimens in the Prague and Miami groups were determined (Fig. 1 through 3). The NT titers of individual sera were higher than the HI titers and were very closely correlated with the HI titers; the correlation coefficients were 0.96 and 0.91 ($P < 0.01$) for the Prague and Miami groups, respectively (Fig. 1). The HI and NT titers of individual sera also showed a significant positive correlation with the SRH zone diameter (Fig. 2 and 3); the correlation coefficients between HI

titer and SRH zone diameter were 0.75 and 0.77 ($P < 0.01$) and those between NT titer and SRH zone diameter were 0.77 and 0.75 ($P < 0.01$) for the Prague and Miami strains, respectively. In our previous study a significant positive correlation between NT and HI titers was demonstrated (11). A significant positive correlation between results of HI and SRH tests has been reported for influenza virus antibodies in horse sera (1), swine sera (5), and human sera (6, 7). The SRH test seems to be particularly suitable for the routine screening of large numbers of serum samples, since it offers the advantage of rapidity and simplicity, although it may be somewhat less sensitive than the HI and NT tests.

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