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# Antigenic Comparison of Swine Influenza Virus Isolates[1]

By

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With 4 Figures

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## Summary

Cross hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests demonstrated strong antigenic relationship between A/Swine/Wisconsin/1/73 (SW/73) and A/Swine/Shope/15/31 (SW/31) influenza viruses. An eigthyone fold purification of virus was achieved by adsorption and elution followed by differential ultracentrifugation and sedimentation through linear sucrose gradient. Radioimmunoassay using purified $^{125}I$ labeled viral antigens revealed antigenic variation between the two virus isolates. Neuraminidase of both viruses had pH optima between 6.5 and 7.0, and SW/31 enzyme was relatively more heat stable than SW/73.

## Introduction

Antigenic drift in influenza viruses involves gradual changes in surface antigens, hemagglutinin (HA) and neuraminidase (NA). The changes may result in mutant viruses possessing altered antigenic sites. *In vitro*, such mutants are known to differ in amino acid sequences of polypeptides (8). In major antigenic shift, complete change in one or both surface antigens due to genetic recombination between influenza viruses of different species of animals may give rise to new viruses (9, 21).

SHOPE (1939), GOMPELS (1953), and JENSEN and PETERSON (1957) did not observe antigenic drift in the HA of swine influenza virus isolates (5, 7, 19). However, MEIER-EWERT *et al.* (1970) demonstrated a slight but significant antigenic drift in both HA and NA (12). This paper describes a study of the antigenic relationship of swine influenza virus isolates of 1931 and 1973.

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## Materials and Methods

### *Growth of Viruses*

Influenza virus strains SW/73 and SW/31 (Hsw 1 N 1) were propagated in allantois of 9- to 10-day-old chicken embryos. Infected allantoic fluid harvested two days after the inoculation of embryos was clarified by centrifuging at 1500 *g* for 15 minutes. Virus was pelleted in an ultracentrifuge (50,000 *g* for 3 hours) and resuspended in 0.2 M sodium phosphate citrate buffer (pH 6.7). Virus was partially purified by adsorption and elution using chicken red blood cells (RBC).

### *Preparation of Antisera*

Hyperimmune sera to viruses were prepared by injecting intraperitoneally 6- to 8-week-old rabbits with 5000 HA units of virus. Every 10 days, the animals were given booster doses intramuscularly with the virus mixed with Freund's incomplete adjuvant[2] and they were bled regularly. Antiserum was inactivated at 56° C for 30 minutes for the neuraminidase inhibition (NI) test and was further treated with Kaolin and chicken RBC for the hemagglutination inhibition (HI) test (17). Sera from rabbits with adequate HI and NI titers were pooled.

### *HA and HI Test*

HA and HI tests were conducted as described by the World Health Organization (22) and adapted to the microtiter method by Sever (18).

### *Assay of Neuraminidase*

Serial two-fold dilutions of virus were made in saline solution (NaCl 0.85 per cent, pH 7.0). Samples (0.05 ml) of each virus dilution (in duplicate) were incubated at 37° C for 30 minutes with 0.05 ml (1.25 mg) fetuin[3] solution in phosphate citrate buffer. N-acetyl-neuraminic acid (NANA) liberated was assayed according to Aminoff (1961). Concentration of neuraminidase giving 0.5 to 0.7 O.D. at 549 mμ was used as standard dilution in the NI test.

### *Neuraminidase Inhibition Test*

Serial two-fold dilutions (0.05 ml) of antiserum in saline (2 tubes/serum dilution) were mixed with an equal volume of standard virus, then incubated at room temperature for 4 hours (20). Duplicate samples of pre-immunization serum from rabbits were included in the test as controls. The neuraminidase activity inhibited, determined by the amount of NANA liberated, was plotted as percentage of activity of the control sample.

### *Preparation of Radioactively Labeled Viral Antigens*

Partially purified virus was sedimented through a 10 to 40 per cent linear sucrose gradient at 35,000 *g* for 45 minutes. One ml fractions were collected and those showing peak HA activity were pooled together, dialyzed against phosphate buffered saline (PBS), concentrated by ultracentrifugation (50,000 *g* for 3 hours), then resuspended in PBS.

Purified virus was dissociated with 2 per cent sodium deoxycholate (DOC) at 37° C for 30 minutes and dialyzed against borate saline buffer (BSB), pH 8.0, then concentrated by evaporation in dialysis tube to a final concentration of more than 3 mg protein/ml. Protein concentration was determined using bovine serum albumin as a standard (10).

The antigen was labeled by mixing it with sodium iodide-$^{125}$I in the presence of iodine monochloride and glycine buffer (11). Unincorporated label was removed by dialysis against glycine buffer (0.2 M, pH 8.6). Antigens were clarified by low speed centrifugation before use.

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[2] Difco Laboratories, Detroit, Michigan.

[3] Grand Island Biological Company, Bethesda, MD.

*Radioimmunoassay*

Various concentrations of unlabeled antigen (0 to 50 μg) and constant amounts of labeled reference antigen (0.25 μg in 5 μl) and antiserum (20 μl) were mixed (duplicate samples) and incubated for 4 hours at 37° C. A predetermined quantity of goat anti-rabbit IgG (0.2 ml) was added; the mixture was further incubated for 1—½ hours at 37° C, and then it was kept overnight at 4° C. The precipitate was twice washed with cold BSB and dissolved in 0.05 M sodium hydroxide. The supernatant and precipitate were counted for 4 minutes and the percentage binding ($^{125}$I antigen) of the uninhibited control sample was plotted against different concentrations of unlabeled antigen. Controls for the experiment were (1) a reaction mixture in which BSB substituted for unlabeled antigen and (2) BSB and normal serum substituted for unlabeled antigen and antiserum.

*pH Optima and Heat Inactivation of Neuraminidase*

Virus samples were incubated with fetuin at different pH values and assayed for neuraminidase activity as described.

Virus was incubated at 45° C in a water bath. Samples were removed at various times and assayed for neuraminidase activity.

## Results

*Purification of Virus*

Adsorption of virus to RBC, followed by its elution and sedimentation, resulted in a 50 per cent recovery of virus HA and 54.8-fold purification on the basis of HA/mg protein (Table 1). An 81.5-fold purification was achieved by sedimentating virus through a sucrose gradient.

Table 1. *Purification of Swine Influenza Virus*[a]

| Sample | Total volume in ml | Total HA ($\times 10^4$) | Percent recovery (HA) | Total protein (mg) | Percent recovery (protein) | HA/mg of protein | Purification (fold) |
|---|---|---|---|---|---|---|---|
| Allantoic fluid | 2000 | 12.8 | 100 | 2000 | 100 | 64 | 1 |
| Eluted virus | 300 | 7.6 | 60 | 228 | 11.4 | 336.8 | 5.2 |
| Sedimented eluate (partially purified) | 4 | 6.4 | 50 | 18.2 | 0.9 | 3508 | 54.8 |
| Sucrose gradient purified | 1.5 | 1.2 | 9.2 | 2.3 | 0.1 | 5217 | 81.5 |

[a] Virus was purified from infected allantoic fluid by absorption and elution on RBC. The eluted virus was further purified by sedimenting through 10—40 per cent linear sucrose gradient, at 35,000 *g* for 45 minutes, and concentrated by centrifugation at 50,000 *g* for 3 hours. HA titer and protein content of each sample were determined to calculate specific activity (HA units/mg of protein).

*Serological Tests*

Table 2 shows that SW/73 and SW/31 antigens cross-reacted in the HI test with antisera. Anti-SW/73 demonstrated stronger cross-reaction between the two antigens than did SW/31 antiserum.

Table 2. *Serological Cross-Reactions between A/Swine/Shope 15/31, and A/Swine/Wisconsin/1/73 in the Hemagglutination Inhibition Test*

| Antiserum | Antigens | |
|---|---|---|
| | A/swine/Wisconsin/1/73 | A/swine/Shope 15/31 |
| A/swine/Wisconsin/1/73 | 1024[a] | 512 |
| A/swine/Shope 15/31 | 640 | 3072 |

[a] Hemagglutination inhibition titers, expressed as reciprocals of serum dilution.

Figures 1 a and b demonstrate a close relationship of SW/73 and SW/31 viruses in NI test. Nevertheless, at 1:40 dilution of anti-SW/73 inhibition of SW/73 neuraminidase activity was 30 per cent greater than that of SW/31. These results, together with the profile of curves in NI test, indicated antigenic differences between the two viruses. Anti-SW/31, however, did not detect any significant difference between the two enzymes (Fig. 1 b).

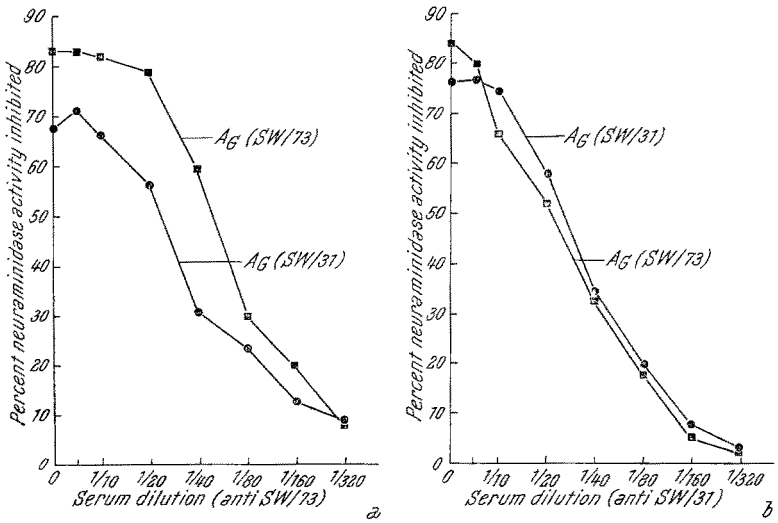


Fig. 1. Inhibition of neuraminidase activity by swine influenza antibodies. Samples of the antiserum and virus mixture were incubated for 4 hours and assayed for neuraminidase activity. AG: Antigen

Antigenic cross-reactions between the two viruses were also observed in the radioimmunoassay (Fig. 2 a and b). Figure 2 a demonstrates that SW/73 antiserum detected antigenic variation between the two antigens. This was indicated by greater inhibition of binding of $^{125}$I SW/73 antigen with SW/73 antiserum when concentrations of unlabeled SW/73 antigen were increased in the test. However, significantly greater quantities of unlabeled heterologous (SW/31) antigen was needed throughout the curve to obtain inhibition comparable to that given by the unlabeled SW/73 preparation.

Figure 2 b demonstrates that anti-SW/31 failed to detect antigenic difference between the two viruses as the curves of displacement of both antigens are not significantly different in inhibition ability.

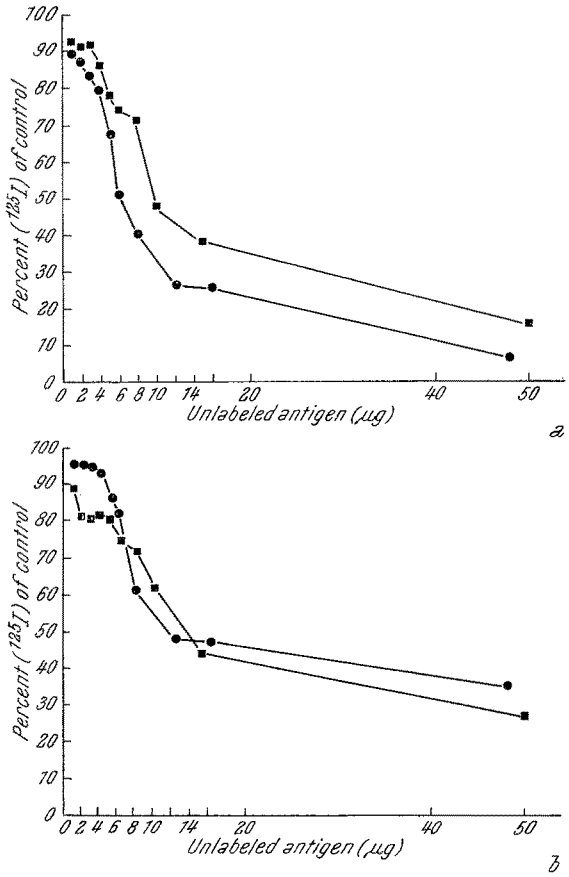


Fig. 2. Binding of $^{125}$I-labeled swine influenza antigen in the presence of unlabeled antigens. a) Binding of $^{125}$I-SW/73 antigen with anti-SW/73; b) Binding of $^{125}$I-SW/31 antigen with anti-SW/31
■———■ SW/31 unlabeled antigen, ●———● SW/73 unlabeled antigen

### *pH Optima and Heat Inactivation Properties*

Liberation of NANA from fetuin as a function of pH is shown in Figure 3. Optimal pH values for SW/73 and SW/31 neuraminidases appear to be in the region of 6.5 to 7.0.

Figure 4 shows that SW/73 neuraminidase was inactivated by 80 per cent in 15 minutes at 45° C. However, SW/31 enzyme was relatively stable; only 30 per cent of the enzyme was inactivated under identical conditions. Further incubation (up to 60 minutes) revealed no change in the heat inactivation property of the two enzymes.

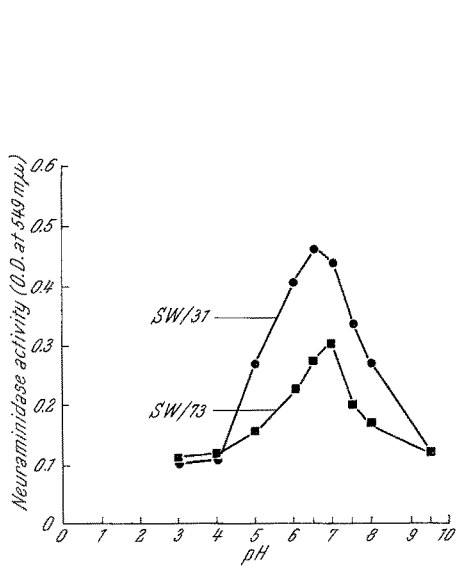


Fig. 3

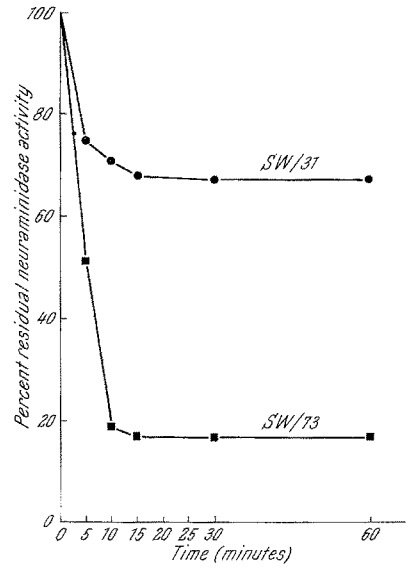


Fig. 4

Fig. 3. pH optima of swine influenza neuraminidase

Fig. 4. Heat stability of swine influenza neuraminidase

## Discussion

Asymmetrical serological cross reactions have been reported by many investigators. NISONOFF *et al.* (13) observed that 30 to 40 per cent of the antibodies to human cytochrome failed to react with rhesus monkey cytochrome; however, in reciprocal cross reaction, both cytochromes reacted identically. This was attributed to the presence of isoleucine in human cytochrome. COCKS and WILSON detected a point mutation by the quantitative microcomplement fixation test in wild type alkaline phosphatase (3). Such asymmetrical cross-reactions were also reported between duck A/Ukraine/63 and human A/Hongkong/71 influenza viruses (9) and between human and swine influenza (2).

PEREIRA *et al.* (15) observed antigenic drift in influenza viruses of birds and horses. Many investigators were unable to show antigenic variation in animal influenza viruses (5, 6, 7, 19). However, slight but significant changes in both surface antigens of swine influenza have been observed (12).

In our studies antigenic variations between SW/73 and SW/31 viruses were revealed in serological tests. However, they were detected only when SW/73 antiserum was used (Figs. 1 and 2). It is possible that SW/31 virus underwent a point mutation, whereby a weak or nonantigenic site was replaced by a strong antigenic site in SW/73. Hence, antibodies to the new antigenic determinant may be produced specific for SW/73 (mutant virus) but unreactive against SW/31.

Similarly, the degree of displacement of $^{125}$I-SW/73 antigen from SW/73 antiserum in the radioimmunoassay by increasing concentrations of unlabeled antigen was greater with SW/73 than with SW/31 antigen (Fig. 2a), indicating that SW/31 antigen lacked new antigenic determinants needed to compete for the antibodies.

However, SW/31 antiserum, which apparently lacked antibodies to new antigenic determinants, did not recognize antigenic difference between the two strains (Fig. 2 b). Likewise, in the NI test an antigenic difference was observed for SW/73 antiserum only (Fig. 1 a and b). Similarly, in the HI test (Table 2). SW/31 antiserum (lacking antibodies to new determinants), when reacted with SW/73 antigen (possessing new determinants), did not inhibit all antigenic sites responsible for hemagglutination. Thus, it demonstrated a relatively low HI titer (20 per cent) as compared to its reaction with SW/31 virus. However, in NI and HI tests, the possibility of steric inhibition by antibodies cannot be excluded.

In the test for pH optima of neuraminidase (Fig. 3), the two viruses did not differ significantly (6.5 to 7.0). Similar results have been reported by DRZENIEK *et al.* (4) for A2 human virus using fetuin as a substrate. PANIKER (14) observed that swine influenza neuraminidase lost 80 per cent of its activity at 45° C. We found that neuraminidase of SW/31 was stable at 45° C (30 per cent inactivation), compared with SW/73 enzyme which showed 80 per cent inactivation (Fig. 4). Differences in the heat inactivation property may possibly reflect changes in the antigenic structure of the two enzymes.

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