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12. Intestinal mucosa (2 g) from one rachitic chick is homogenized in 25 ml of 0.25*M* sucrose in 0.05*M* tris-HCl (*p*H 7.4), 0.25*M* KCl, and 0.005*M* $MgCl_2$. The cytosol fraction is obtained by centrifugation at 100,000*g* for 1 hour. Chromatin is prepared from crude nuclei (isolated from the original homogenate by centrifugation at 1000*g* for 10 minutes) by homogenizing them successively in one 25-ml portion of 0.8 m*M* EDTA, 25 m*M* NaCl, *p*H 8; one 25-ml portion of 1 percent Triton X-100, 0.01*M* tris-HCl, *p*H 7.5; and one 25-ml portion of 0.01*M* tris-HCl, *p*H 7.5. The chromatin is harvested by sedimentation at 30,000*g* for 10 minutes after each wash. The entire chromatin pellet from 2 g of mucosa is reconstituted with half the cytosol fraction by homogenization to create a cytosol-chromatin receptor system for the competitive binding assay. The reconstituted homogenate is then forced through a 22-gauge needle. All operations are performed at 0 to 4°C, and receptor system is prepared immediately prior to use. To each assay tube containing tritiated 1,25-$(OH)_2D_3$ and unlabeled sterol (dried together with a stream of nitrogen) is added 10 μl of distilled ethanol and 100 μl of the reconstituted cytosol-chromatin system (containing about 100 μg of DNA). The final concentration of tritiated 1,25-$(OH)_2D_3$ is 4.3 n*M*. After incubation for 30 minutes at 25°C with vigorous shaking in a water bath, the quantity of labeled sterol bound to chromatin is determined by filtration. To each assay tube, 1 ml of cold 1 percent Triton X-100 in 0.01*M* tris, *p*H 7.5, is added and the entire mixture applied to a Gelman Type A/E glass fiber filter at very low vacuum. After 2 to 4 minutes the vacuum is increased to achieve uniform flow rates of about 1 ml per minute, and each of the filters is washed with 2 ml of 1 percent Triton X-100 and 0.01*M* tris, *p*H 7.5. After filtration, the filters are placed in liquid scintillation vials with 5 ml of methanol-chloroform mixture (2 : 1 by volume). After 20 minutes the methanol-chloroform mixture is evaporated and the sterols are solubilized in a standard toluene base mixture and counted according to liquid scintillation procedures. Sterol sources were as follows: nonradioactive 25-$(OH)D_3$, [Dr. J. Hinman] Upjohn; tritiated 25-$(OH)D_3$ (6 to 8 c/mmole), Amersham/Searle; nonradioactive 1,25-$(OH)_2D_3$, [Dr. M. Uskokovic] Hoffman La Roche; the tritiated 1,25-$(OH)_2D_3$ was generated enzymatically from 25-$(OH)D_3$ and purified as described by P. F. Brumbaugh and D. H. Haussler [*J. Biol. Chem.* **249**, 1251 (1974)].
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## Persistence of Hong Kong Influenza Virus Variants in Pigs

Abstract. *The A/Hong Kong/1/68 (H3N2) influenza virus which has not been isolated from man for several years, was recently isolated from pigs in Hong Kong. Influenza viruses similar to A/Victoria/3/75, which are currently circulating in man, were also isolated from pigs. Both above-mentioned viruses could be transmitted readily from pig to pig in experimental studies. The isolation of influenza viruses similar to A/Hong Kong/68 from pigs in 1976 suggests that pigs may serve as a potential reservoir for future human pandemics as well as a possible source of genetic information for recombination between human and porcine strains of influenza virus.*

Influenza viruses antigenically identical with the A/Hong Kong/1/68 (H3N2) strain from man were first isolated from slaughterhouse pigs in Taiwan in 1969 (*1*). Serological studies have shown that Hong Kong/68 influenza virus appeared in man in 1968 prior to infection of pigs with this virus (*2, 3*). The detection of antibodies to Hong Kong influenza virus in pigs in widely separated parts of the world—Germany, Hungary, Great Britain, United States, and Taiwan (*2, 3, 4*)—suggests either that this virus spreads rapidly in pigs or, more likely, that multiple transmissions from man to pigs have occurred. The Hong Kong/68 influenza virus caused no overt signs of disease in pigs, and experimental transmission to contact animals could only be demonstrated serologically (*1, 3*). Serological and virus isolation studies since 1972 indicate that the A/England/42/72 and the A/Port Chalmers/1/73 variants of Hong Kong/68 influenza virus have also been transmitted to pigs and avian species (*5, 6*). There has been no evidence to date that the Hong Kong strains have been maintained in the pig populations of the world.

We now describe (i) the characterization of A/Hong Kong/1/68–like and A/Victoria/3/75–like influenza viruses isolated from pigs in Hong Kong in 1976 and (ii) experimental transmission studies of these viruses in pigs. In man, the A/Hong Kong/1/68 variant of influenza virus has been superseded by the England, Port Chalmers, Scotland, and Victoria strains

Table 1. Identification of influenza viruses isolated from pigs in Hong Kong in May 1976 by hemagglutination inhibition. Postinfection ferret serums were provided by the Center for Disease Control Atlanta; the serums were treated with receptor-destroying enzyme and tested in HI assays with four agglutinating doses of virus as described (*8*).

| Virus | HI titers with postinfection serums to: | | | | | | |
|---|---|---|---|---|---|---|---|
| | Hong Kong/68 (F)* | England/72 (F) | Port Chalmers/73 (F) | Scotland/74 (F) | Victoria/75 (F) | Sw/HK/3/76 (P)* | Sw/HK/4/76 (P) |
| A/Hong Kong/1/68 | 6,000 | 1,600 | 300 | 200 | 560 | 160 | <20 |
| A/England/42/72 | 1,000 | 18,000 | 2,000 | 400 | 560 | 40 | <20 |
| A/Port Chalmers/1/73 | 150 | 2,000 | 2,000 | 400 | 300 | 20 | 20 |
| A/Scotland/840/74 | <20 | 300 | 560 | 2,000 | 150 | 20 | <20 |
| A/Victoria/3/75 | <20 | 640 | 4,500 | 150 | 18,000 | <20 | 640 |
| A/swine/HK/3/76 | 2,500 | 6,400 | 300 | 150 | 500 | 160 | <20 |
| A/swine/HK/4/76 | <20 | 300 | 1,000 | <20 | 4,500 | <20 | 320 |

*F = ferret serums, P = postinfection pig serums. The figures represent the reciprocals of the serum dilutions inhibiting three out of four agglutinating doses of virus.

Table 2. Identification of the neuraminidase on influenza viruses isolated from pigs in Hong Kong in May 1976. Hyperimmune rabbit antiserum to the isolated neuraminidase subunits of the influenza viruses indicated were used in the neuraminidase inhibition test recommended by the World Health Organization (*9*). The figures represent the reciprocals of the serum dilutions inhibiting 50 percent of the neuraminidase activity.

| Virus | NI titers to the following antiserums: | |
|---|---|---|
| | A/Hong Kong/1/68 | A/Victoria/3/75 |
| A/Hong Kong/1/68 | 650 | 100 |
| A/England/42/72 | 260 | 180 |
| A/Victoria/3/75 | 10 | 200 |
| A/swine/HK/3/76 | 100 | 10 |
| A/swine/HK/4/76 | <10 | 200 |

Table 3. Virus isolation and signs of disease in experimentally infected pigs. For each influenza virus strain, two young 40-pound pigs were inoculated intranasally with approximately $10^6$ $EID_{50}$ of third-egg-passage influenza virus and housed in separate isolation facilities at Plum Island Animal Disease Center. Five hours later four contact pigs were introduced. Nasal swabs and temperatures were taken daily. The swab material was inoculated into 10- to 11-day-old chick embryos, and influenza virus was detected by hemagglutination.

| Virus strain | Designation and number of pigs inoculated | Virus isolation/ days after inoculation | Signs of disease |
|---|---|---|---|
| A/swine/HK/3/76 | Inoculated (2) | 1 to 4<br>1 to 5 | Heavy nasal discharge in one on days 5 and 6 |
| | Contact (4) | 7 to 11 | Mild cough on day 10 |
| A/swine/HK/4/76 | Inoculated (2) | 1 to 9<br>1 to 8 | No signs |
| | Contact (4) | 2 to 9, 3 to 9,<br>3 to 12, 4 to 12 | No signs |

and has not been isolated from man for a number of years.

Isolation of a swinelike influenza virus from man (*7*) [A/New Jersey/8/76 (Hswl N1)] has led to increased surveillance of pig populations throughout the world for the isolation of influenza viruses. Tracheal swab material collected at random from 356 apparently normal pigs from an unknown number of herds in a slaughterhouse in Hong Kong during May 1976 were inoculated into 10- to 11-day-old chick embryos by the allantoic route. A total of 11 influenza viruses was isolated and characterized in hemagglutination inhibition (HI) tests and neuraminidase inhibition (NI) tests. Six of the isolates (illustrated by A/swine/HK/3/76 in Tables 1 and 2) reacted strongly with antiserums to the prototype A/Hong Kong/1/68 strain both in HI and NI tests. The other five isolates (illustrated by A/swine/HK/4/76 in Tables 1 and 2) reacted to higher titers with antiserums to the recent A/Victoria/3/75 strains. The slight differences between the A/Hong Kong/1/ 68 human strain and the A/swine/HK/3/76 strain in HI tests were found with all six isolates when postinfection ferret serums were used but were not apparent when postinfection pig serums for A/swine/HK/3/76 were used.

Experimental infection of pigs with A/swine/HK/3/76 showed that virus was recovered from the inoculated animals on each of the first 4 or 5 days after infection; virus was recovered from each of the four contact pigs, the first isolations being made 7 days after they were put in contact with the inoculated animals. One of the inoculated pigs had a heavy nasal discharge on days 5 and 6 after inoculation, and the contact animals developed a mild cough on day 10.

Rectal temperatures of 38.2° to 40.5°C were recorded and may reflect agitation of the animals rather than disease signs; one of the contact pigs showed a definite febrile response (41.3°C) on day 10; this response corresponded to the time of virus isolation and coughing in that animal. All the pigs inoculated or in contact with A/swine/HK/3/76 developed HI antibodies with titers up to 1 : 160 with both homologous virus and A/Hong Kong/1/68. Serums from these pigs after infection failed to inhibit A/Victoria/3/75 influenza virus (Table 1).

Virus was recovered from pigs inoculated with A/swine/HK/4/76 from 1 through 9 days after inoculation and from contact pigs from 2 through 12 days after they were put in contact with the inoculated animals (Table 3). Clinical signs were not observed in inoculated or contact pigs. All of the pigs developed antibody; the 21-day serum HI titers were up to 1 : 320 with homologous virus and 1 : 640 with A/Victoria/3/75. Postinfection serums from these pigs failed to inhibit A/Hong Kong/1/68 influenza virus (Table 1). The A/swine/HK/3/76 and A/swine/HK/4/76 were transmitted to all contact pigs but produced mild or no clinical disease signs.

The isolation from pigs, in 1976, of Hong Kong influenza virus variants that are no longer circulating in the human population suggests that this virus may have become established in the pig populations of Asia. Since human influenza viruses were not handled in the laboratory where the above viruses were isolated, it is very unlikely that these viruses represent laboratory contaminants. Viruses related to swine influenza (Hswl N1) were not isolated although our limited study does not exclude the possibility of the existence of the virus in this area of the world. A limited serological survey with 200 of the 356 pigs sampled showed HI antibodies to the A/swine/HK/4/76 virus in 15 percent of pigs, antibodies to A/swine/HK/3/76 in 0.5 percent of pigs, but antibodies to swine influenza virus (A/New Jersey/76) were not detected. The low incidence of antibodies to the A/swine/HK/3/76 strain probably reflects recent infection in some of the 356 pigs sampled.

The persistence of Hong Kong influenza virus variants in pigs could serve as a reservoir for subsequent infection of man, as well as increasing the possibility of antigenic variation by recombination in nature with the influenza viruses (Hswl N1) already in pigs. The other possibility is that antigenic drift of human strains might occur in pigs, but this has not occurred rapidly in the classical swine influenza virus strains.

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**References and Notes**

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