SERO-EPIDEMIOLOGY OF INFLUENZA IN MAN AND ANIMALS*

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I. Tracing the spread of England /72 Influenza in people of Kathmandu, Pokhara and Langtang.

A new variant of influenza virus was isolated in Coonoor, South India in July 1971. The virus is called A/England/42/72 (H₃N₂); this is abbreviated as England/72. England/72 and the last epidemic strain A/Hong Kong/1/68 (H₃N₂) or Hong Kong/68 are antigenically closely related. The new variant is now causing epidemics throughout the world. (Schild et al, 1972).

Since England/72 and Hong Kong/68 are close relatives, the tests for antibody are interpreted in the light of their known antigenic relationships. In the hemagglutination inhibition (H-I) tests both viruses cross react with a proportion of the antibody stimulated by the other. Using post-infection ferret sera England/72 will detect 1/16 of the antibody stimulated by Hong Kong/68 and Hong Kong/68 will detect 1/4 of the antibody stimulated by England/72 (Schild et al, 1972). If human sera behave like post-infection ferret sera, tests for Hong Kong/68 antibody may be positive if a serum contained England/72 antibody (see 0-9 age group, Table 1). If a serum contains Hong Kong/68 antibody, the levels must be high before England/72 virus will detect sufficient quantities so that it may be considered positive. An antibody titer of 1/40 or greater is considered positive in the H-I test.

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Another consideration in interpreting H-I test results is the "doctrine of original antigenic sin." The doctrine states that if a new variant virus penetrates a population and the new virus is immunologically related to one that previously infected this population, antibodies will be produced against the new variant as well as against the virus which was originally present. Thus, in the present instance England/72 may not only stimulate the production of antibodies against itself but also against Hong Kong/68. The net result is that the prevalence and geometric mean titer (GMT) of Hong Kong/68 will be higher than if they resulted from prior Hong Kong/68 infection. To determine if England/72 or Hong Kong/68 is circulating in the population the GMT is the most reliable measurement.

To determine if England/72 has penetrated Nepal, antibody levels were determined in sera drawn from 328 residents of Kathmandu during August through September 1972 and from 105 residents during late November 1972 and through early January 1973. The donors were a composite of outpatients and inpatients, professional blood donors, and maternity cases. The sera of 57 adults in Langtang village and 47 adults in Pokhara were also tested (Table 1).

In Kathmandu England/72 had infected 57% of the people tested by August and September 1972. The GMT for all ages combined was 164. At this time the children from 6-9 years of age had a 72% (23/32) prevalence of infection and a GMT of 947. The apparent prevalence of Hong Kong/68 in these same children was 66% (21/32) and the GMT was 246. However, since Hong Kong/68 has the capacity to cross react with 25% of the England/72 antibody, the prevalence of Hong Kong/68 must be considerably lower than 66%. In the older people the prevalence and GMT of England/72 and Hong Kong/68 antibodies declined. However, one age group was without England/72 antibodies. If the doctrine of original antigenic sin was operative the Hong Kong/68 antibody may be higher in titer, at least in the older age groups, than England/72. That antibody levels to Hong Kong/68 antibody were consistently lower than England/72 suggests that Hong Kong/68 may provide some protection against England/72 infections in people 10-49 years of age (Table 1).

To measure the rate of dissemination of England/72 infections in Kathmandu the sera of 105 people drawn 3½ months after the initial sample of 328 were tested for H-I antibody. The overall prevalence of England/72 in the group of 105 was 77% (81/105) and the GMT was 284. During the 3½ months period the prevalence of England/72 infections increased from 57 to 77%. Similarly, the GMT increased from 164 to 284. In contrast the prevalence of Hong Kong/68 increased from 68 to 71%, and the GMT increased from 96 to 138. Again, these increases in the prevalence and GMT of Hong Kong/68 are in all likelihood due to cross reactions with England/72 antibody.

Pokhara, a city 100 miles from Kathmandu having an elevation of 2500 ft. served as a geographic comparison. Sera drawn from adults during early January showed a prevalence of 68% (32/47) and a GMT of 167 for England/72. Hong Kong/68 prevalence was 43% (20/47) with a GMT of 54. Langtang village, located at 12,000 ft. elevation and 20

minutes by air from Kathmandu also served as a geographic comparison. Tests on sera drawn during the second week of October showed an overall prevalence of 30% and a GMT of 77% for England/72. The prevalence and GMT for Hong Kong/68 was 32% and 59 respectively. At the same time, Kathmandu was experiencing 57% prevalence of England/72 (Table 1).

Using the 57% and 77% England/72 prevalence figures derived from the Kathmandu population and extrapolating to sera time it would appear that England/72 entered Kathmandu during the first part of October 1971 three months after the virus was isolated in Coonoor, India. When compared with Kathmandu, both the prevalence and GMT in Pokhara were lower in January 1973. The lowest prevalence and GMT recorded was in the people of the more isolated Langtang village. Thus, England/72 virus first penetrated Kathmandu then Pokhara and lastly Langtang village. Forty six percent of the sera (22/48) drawn during September 1971 through January 1972 from children in Calcutta contained antibodies for England/72. If the new variant virus made its first appearance in Coonoor, India, its spread to Calcutta and Kathmandu was extremely rapid. Perhaps England/72 appeared in Asia much before July, 1971.

Based on its prevalence, GMT and the possibility that Hong Kong/68 antibody may provide some protection against severe England/72 infections, an epidemic in Langtang village should be accompanied by a greater morbidity and mortality. Interviews with the people in Langtang village during December 10-15, 1972 (three months after the sera was collected) revealed many complaints of fever, muscle pain, headach and respiratory symptoms during October and November, 1972. However, regarding the Kathmandu population, a retrospective recommendation for vaccination with England/72 in the 5 age groups studied would be only for the children 0-9 years of age (Table 1). By extrapolation the England/72 epidemic should be over in Kathmandu in May, 1973.

II. A Search for animal reservoirs of influenza.

For unknown reasons the last three variants of influenza virus which caused epidemics throughout the world were first isolated in South-East Asia. Chronologically these variants were A/Singapore/1/57 (H₂N₂), Hong Kong/68 and England/72. Either animals or men both could have been the host for these epidemic variant viruses. It is known that experimentally an equine virus called A/Equine/Miami/1/63 (Hq₂ Nq₂) (Miami 63) can infect humans and Hong Kong/68 can infect horses (Kasel and Couch, 1969). Under natural conditions, Hong Kong/68 has been isolated from a pig (Mundin, 1970) and a sick chicken (Zhezmer, 1973). The close antigenic relationship between Hong Kong/68 and Miami/63, is well established (Tumova and Easterday, 1969). Clearly, the species which was the actual host for these new variant viruses has not been determined.

Tests were conducted to examine whether England/72 and Hong Kong/68 have infected animals and whether Miami/63 and another equine virus called A/Equine/Pra-

gues, 1/56 (Heq₁ Neq₁) Prague/56) and the avian viruses A/Turkey/Mass/65 (Hav₆ N₂) (Turkey/65) A/Quail Italy/1117/65 (Hav₂ Neq₁) (Quail/65) and A/Duck/England/56 (Hav₃ N₁) (Duck/56) have infected humans in Nepal. Of 134 human sera tested with avian influenza antigens 3 children had low levels (1/40, 1/80, 1/160) of antibody against Quail/65 virus. All three children were in the 0-2 year age group and were hospitalized in the Kanti Hospital. The other tests with avian antigens were negative. All of 540 human sera were negative for Prague/56 antibodies. Experimentally it has been shown that Miami 63 antigen will cross react with 50% of antibody stimulated by Hong Kong/68. (Tumova and Easterday, 1969). As expected, Miami 63 antigen detected low levels of antibodies in 12% (37/318) of the human sera tested. The prevalence of Miami 63 in people of Langtang village and Kathmandu was similar. In all probability these data indicate cross reactions of Miami/63 antigen with Hong Kong 68 antibody rather than primary infections of people with this equine virus.

Equines could be a host for new variant viruses, therefore the sera of 36 horses and 20 mules were tested for H-I antibodies using the following equine and human antigens Prague/56, Miami/63, England/72 and Hong Kong/68. Antibody for Prague/56 was found in one 13 years old horse which was stabled in Calcutta during the epidemic of equine influenza which occurred in Bombay and Calcutta in 1954. Also three 9 years old Tibetan horses which were born in Nepal and presently residing in Lumle had low levels (1/40) of Prague/56 antibody. No Miami/63 antibody was found in any of the horses or mules tested.

All mules, and all except one horse had high levels of an H-I inhibiting factor for England/72 and for Hong Kong/68 viruses. However, equine sera frequently contain substances which behave like antibody and give false positive H-I tests. Receptor destroying enzyme (RDE) which was routinely used to destroy inhibitors in all sera before testing for H-I antibodies reduced the concentrations of these inhibitors. Frequently trypsin-heat (50°, 30 min)-KIO₄ treatment is more effective than RDE and regarding equine sera, KIO₄ alone will reduce the concentration of these inhibitors. Following KIO₄ treatment H-I inhibiting factors for England/72 and Hong Kong/68 were found in 70% (39/56) and 66% (37/56) of the equine sera tested. The GMT for England/72 was 205 and for Hong Kong/68 the GMT was 133. In ovo, 5 of 6 equine sera at dilutions of 1/40 neutralized 200 eggs infectious units of England/72 virus. Immuno-diffusion tests for Hong Kong/68 and England/72 antibody in equine sera showed rings of opalescence after placing the sera in gels containing the antigens. However, the opalescence disappeared within 7 to 10 days. Precipitates containing hyperimmune sera and antigens do not disappear. Additional tests demonstrating lines of identity between equine sera and hyperimmune sera containing only England/72 or Hong Kong/68 antibody should prove or disprove whether the equine in Nepal and Calcutta have been infected with either or both of these viruses. If antibody is found in equine sera the question is "did humans infect horses or vice versa."

To identify additional possible reservoirs of influenza, 47 equine sera were tested for avian influenza H-I antibodies. The antigens used were Duck/56, Quail/65, Turkey/65 and A/chicken/Scotland/59 (Hav₆ N₁). No antibodies were found. Similarly, sera from crows trapped in Barasat, a city 14 miles from Calcutta, were tested with Newcastle disease viruses (NDV) as well as with all of the previously mentioned antigens. Fifteen percent (27/185) of the crows had NDV antibodies and the GMT for NDV was 86. Hong Kong/68 antibodies or H-I inhibiting factors at titer of 1/40 or greater were detected in 55% (101/185) of the crows. The GMT was 63. If proof is obtained that crow sera contain specific antibody for Hong Kong/68 virus a new avian reservoir has been discovered. Also, 3 yaks were bled during December 1972 in Langtang village. The sera were tested with all of the antigens previously listed as well as parainfluenza type 3. Antibodies or inhibiting factors for Hong Kong/68 virus were found in two of the animals. If the antibodies are specific for Hong Kong/68 a new bovine host for the virus has been identified.

The new epidemic virus England/72 was detected not only in Coonoor, India. In May and June 1972 the virus was also isolated during influenza epidemics in Malaysia, Singapore and Australia (Schild et al, 1972). Monkeys are common to all 4 geographic areas. Therefore the sera of 43 free-living long-tailed macaques (*Macacus fascicularis*) and 2 leaf monkeys (*Presbytis cristatus*) which were trapped in a mangrove swamp forest on Carey Island, Malaysia during 1969 and 1970 were tested for H-I antibodies. serial serum samples from 5 of the monkeys which were captured more than once over a 13 month period provided an opportunity to measure decreases in antibody levels and serological conversions. All of the antigens previously listed were used in the tests.

The prevalence of England/72 antibodies or H-I inhibiting factors in the monkeys was 88% (37/42) and the GMT was 339. Hong Kong/68 had a prevalence of 98% (41/42) but the GMT was 133. Evidence was also obtained for an infection with the equine virus Prague/56 or some relative of this strain of influenza which has not been as yet, isolated and characterized. The prevalence and GMT for Prague/56 was 50% (21/42) and 300 respectively. Transient areas of opalescence lasting 24-48 hours were produced with 5 of 50 sera in immuno-diffusion plates containing England/72 antigen but none were observed with Hong Kong/68 antigen.

H-I tests with serum samples drawn from 5 monkeys in February 1969 through February 1970 showed a decreasing England/72 titer in 3 monkeys, a constant titer in one 5 years old female. This serological conversions demonstrates that England/72 or a close relative was circulating among the long-tailed macaques of Carey Island, Malaysia in June of 1969 which was 25 months prior to the first isolation of the virus from human. However the declining titers indicate that England/72 infections also occurred prior to February 1969. Since long-tailed macaques as well as the 2 leaf monkeys had England/72 antibodies the infection was not confined to one species group. Few people inhabit the area where the monkeys were trapped and the 2 leaf monkeys were shot by the aborigines with darts dipped

in ipoh poison. All monkeys were bled at the site of capture and released after bleeding. Thus it seems unlikely that humans were the source of the England/72 virus. This new epidemic strain may have originated in monkeys or the monkeys were infected by some other animal on Carey Island much before the virus was isolated from humans in Coonoor, India. Environmental control of influenza awaits the discovery of the species which is the host for the new epidemic strains. It would appear that monkeys are likely candidates and that a jungle cycle of influenza is present in nature.

Table 1

Prevalence and geometric mean titer of England/72 and Hong Kong/68 H-I antibodies in Kathmandu, Pokhara and Langtang village.

Sera drawn during August through September 1972 in Kathmandu.

Age (years)	Geometric mean titer		Prevalence (%)	
	England/72	Hong Kong/68	England/72	Hong Kong/68
0-9	947	246	72 ($\frac{23}{32}$)	66 ($\frac{21}{32}$)
10-19	193	80	63 ($\frac{26}{41}$)	71 ($\frac{29}{41}$)
20-29	126	84	56 ($\frac{100}{177}$)	71 ($\frac{126}{177}$)
30-39	89	89	49 ($\frac{29}{59}$)	56 ($\frac{33}{59}$)
40-49	190	114	53 ($\frac{8}{16}$)	73 ($\frac{11}{15}$)
All ages	164	96	57 ($\frac{188}{328}$)	68 ($\frac{223}{328}$)

(continued...)

Table 1 (Continued)

Sera drawn during late November through early January in Kathmandu.

Age (year)	Geometric mean titer		Prevalence (%)	
	England/72	Hong Kong/68	England/72	Hong/Kong/68
0-9	453	74	63(10/16)	56(9/16)
10-19	525	238	78(7/9)	78(7/9)
20-29	218	146	83(43/52)	73(38/52)
30-39	320	143	75(18/24)	75(18/24)
40-49	320	101	75(3/4)	75(3/4)
All ages	284	138	77 (81/105)	71(76/105)

Sera drawn during December 1972 and early January 1973 in Pokhara.

10-68	167	54	68(32/47)	43(20/47)
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Sera drawn in October 1972 in Langtang village.

11-90	77	59	30(17/57)	32(18/57)
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