APPLICATION OF SEROEPIDEMIOLOGY TO PROSPECTIVE STUDIES OF AVIAN INFLUENZA

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SUMMARY

This study was conducted to evaluate the usefulness of the single radial haemolysis (SRH) technique for the detection of antibodies to avian influenza viruses in sera from people in the geographic regions of Pearl River Delta and Taiwan. Antibodies were found to all known avian virus haemagglutinin subtypes and populations from rural areas showed different extents of exposure from those in urban environments ($p<0.05$). Additionally, people from the rural areas showed evidence of multiple infection by these avian influenza viruses. The importance of these findings is discussed in the context of the hypothesis that human pandemic influenza has its origin in a non-human species.

The fact that viruses of a particular subtype are more frequently isolated from animals especially ducks is H4, and in this study antibody to the H4 subtypes were jointly the most common antibodies (except H3) detected occurring in 10% of the rural area sera.

INTRODUCTION

Influenza is unique among infectious agents affecting man in its ability to change periodically the antigens on its surface, so that the immunity produced by one strain provides little or no protection against strains which subsequently arise. Because of this influenza continues to be a major epidemic disease of man. Although great advances have been made in recent years in understanding antigenic variation of influenza viruses at the molecular level, we are still no closer to predicting or preventing the emergence of pandemics than we were fifty years ago. Primarily, this is because we neither know with any degree of certainty the likely geographic source nor the reservior from which they might emerge. However, evidence base on animal influenza viruses, arising from the

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hypothesis of Webster and Laver (1), have result in the isolation of a wide varity of avian influenza viruses in the domestic poultry of Pearl River Delta.

Factor Giving Rise to the Hypothesis that Pandemic Strains are Derived From an Animal:

Comparative serological studies conducted soon after the emergence of the Hong Kong virus showed that its HA was closely related antigenically to those of the animal viruses A/duck/Ukraine/63 (H3N8) and A/equine/Miami/63 (H3N8), that had been isolated some five years prior to the H3N2 virus (2) (3) (4). Tryptic peptide mapping studies soon confirmed biochemically this relationship, particularly with the duck virus (5), that was consolidated in sequence studies some years.

These studies were conducted in the knowledge that serological data obtained some years earlier indicated that the virus caused the 1918-1919 pandemic was antigenically related to a viurs apparently persisting in pigs in the United States. Subsequent studies showed that this H1N1 virus continued to be present in the United States and in Southeast Asia (6) (7). Indeed the only definitive evidence of human infection by animal virus comes from a number of incidents attributable to the swine (H1N1) virus, the most publicised being the one at Fort Dix, New Jersey. Furthermore, H1N1 viruses obtained from turkeys in the U.S.A. that were closely related to many respects to the H1N1 virus prevailing in pigs caused influenza in a technician handling the turkeys (8). Recently, a virus isolated from sick harbour seals with "avian" antigens caused conjunctivitis in investigators during experimental studies (9). It is upon data such as these considered here that the hypothesis for the involvement of a non-human viruses in the emergence of pandemic influenza lies. It is also the basis of this study which seeks to explore human exposure to such viruses.

Factors in the Region of Pearl River Delta that Might Favour Human Exposure to Avian Viruses:

Pearl River Delta is basically a region of mountains and the small proportion that is available for farming has to support a huge human population. To do this, the arable land has been intensively cultivated in the most efficient means possible for the last two millennia up to present times using age old farming methods (except Taiwan). The most economical way of doing this is to use cereals and vegetables directly.

Under these circumstances, ducks are raised in large numbers as an adjunct to rice farming. Ducklings feed to a large extent on live food after the rice has firmly taken root in the flooded while birds at the finishing stage of growth, around 70 to 80 days, feed on the fallen grain. This type of duck raising does not compete to any extent with pigs and other livestock for cereal and accommodates the need to conserve as much grain as possible for direct human consumption.

Thus, one might envisage amplification of virus in the duck populations, particularly in susceptible ducklings, newly introduced into the flooded rice fields at the time of the spring planting, by waterborne infection (10) arising from the faecal contamination of the water by a few "carrier" ducks. Such a situation would lead to an increase in the occurrence of virus in the environment in the warmer months available for transmission

to man.

The millieu that exists in the numerous villages and communities in this region, the main rice farming area of the country, bring man and his animals into very close association and it is not inconceivable that in this environment the opportunity for interspecies transmission would be considerable. These views are based on ideas arising from meetings in Hong Kong and Peiking (11).

Sera made available from farmers in the Pearl River Delta and from Taiwan were used as the basis of the seriological approach for evaluating human exposure to avian viruses. While it could not be ascertained that the farmers were specifically duck farmers, the very fact that they live in localities were ducks are raised and rice grown was considered to be more than adequate for the purpose of the study. That being so, the serological study was undertaken on the premise that (1) exposure to a "purely avian" viruses devoid of the genes for virulence and transmission in man would provide sufficient antigenic stimulus leading to antibody production and (2) given the nature and intensity of the agricultural ecosystems, particularly those in the Pearl River Delta, repeated exposure to avian viruses from time to time is likely and would enhance antibody production in the event that the antigenic stimulus provided was minimal or poor.

Thus, sera from farmers in the two localities were submitted to an examination for the presence of antibodies to viruses covering the full range of known avian HA subtypes, namely H3 to H13, covering the range previously designated Hav1 to Hav10 and a newly recognised subtype, H13. The serological method of initial choice was single radial haemolysis and the rationale for its use is outlined by Kendall (12).

As an epidemiological control, sera from dwellers in urban Hong Kong, a place with perhaps the highest population spot density in the world, were included on the grounds that such people were unlikely to have been exposed to viruses other than those prevailing in man. Sera from people in Urban Taichung were included as a control for the farmers of Taiwan.

MATERIALS & METHODS

Viruses:

All the avian influenza A viruses except those marked with an asterisk were isolated from poultry in Hong Kong in the course of surveillance studies by K.F. Shortridge. The first group of viruses used for the study were chosen for their affinity with reference monospecific antisera in the HI test because they had a neuraminidases different from those of the human H1N1 and H3N2 viruses in an attempt to avoid the possibility of detection of antibodies to these antigens.

Human Sera:

The number of sera used in this survey was arrived at by a compromise between the amount of sera available and the time required to complete the study. Because of the findings of the virus surveillance studies, sera from the Pearl River Delta area were

Virus	Designation	Antigenic Combination
A/duck/HK/526/79	D526	H3N6 (Hav7Nav1)
A/duck/HK/668/79	D668	H4N5 (Hav4Nav5)
A/duck/HK/313/78	D313	H5N3 (Hav5Nav2)
A/duck/HK/531/79	D531	H6N8 (Hav6Neq2)
A/duck/HK/47/76	D47	H7N2 (Hav1N2)
*A/turkey/Ont/6118/68	–	H8N4 (Hav8Nav4)
A/duck/HK/147/77	D147	H9N6 (Hav9Nav1)
A/duck/HK/876/80	D876	H10N3 (Hav2Nav2)
A/duck/HK/661/79	D661	H11N3 (Hav3Nav2)
A/duck/HK/862/80	D862	H12N5 (Hav10Nav5)
*A/gull/Md/704/77	–	H13N5

considered to be potentially the most interesting and thus comprised the largest number. They were made available by M.H. Ng, Department of Microbiology, University of Hong Kong, from a study on nasopharynageal carcinoma. As far as we know all the sera from the Delta area came from rural dwellers or farmers. The 100 sera comprising the Hong Kong group were kindly provided by D. Robinson, Biochemistry Department, University of Hong Kong, who collected them in November 1982 from people living in the urban enviroment of Hong Kong Island. The 190 sera from rural Taichung, Taiwan we collected in November 1982, and we were able to establish that all these came from farming people.

The sera from urban Taichung, Taiwan numbered 95 and these we also collected in November 1982. Because Taichung is a fast growing city and there is extensive migration from the rural areas to the urban area, it was not possible to eliminate from this group those persons who had recently moved into the city. Thus, there may be a number of sera in this group from such persons.

Preparation of Single Radial Haemolysis Immunoplates and Test Procedure: (12)

1. Influenza virus in crude allantoic fluid were added in 10%(v/v) chicken red blood cell at a standard concentration of 500HAU/ml in phosphate buffered saline (pH = 7.0 – 7.2) and held at 4°C for 10 min (virus absorption).
2. Unabsorbed virus was then removed by two cycles of low speed centrifugation at 4°C.
3. 0.3 ml of virus treated (10%) chicken RBC was incorporated into 3.0 ml of (1%) melted A-37 Indubiose agarose in PBS in 42°C water bath.
4. Mixed the suspension quickly, and poured into Falcon 3002 tissue culture dishes, then dried at room temperature for 30 min and 2.8mm wells were punched in each plate.
5. 5 micro-liters of heat treated serum (60°C for 20 min) were delivered into two wells on opposite side of one plate to diffuse for 30 min.
6. 5 micro-liters of complement (dilute guinea pig serum) were added to each well,

then incubated overnight at 37°C.

7. Examined for evidence of haemolysis.

RESULTS

A total of 785 sera from four areas or localities in the region was examined for evidence of avian virus infection and the results summarized by HA subtype in Table 1.

45% of the sera were positive for the avian H3 subtype but such data do not allow differentiation between antibody due to avian or human H3 infection or both. Of the other subtypes, namely H4 to H13, the occurrence of seropositivity was not greater than 9% for any one of them.

The diameters of the zones of haemolysis ranged from 3mm to 7.3mm. Haemolysis was recorded for all subtypes at the lower limit considered as a positive reaction is 3mm. The mean diameters ranged from 4.1mm for the H11 subtype to 4.8mm for the avian H3 subtype. Although haemolysis for the H5 strains subtype is the least common (1%) the mean diameter was 4.5mm, whereas haemolysis for the H11 subtype, occurring in 9% of the samples had a mean diameter of only 4.1mm. However the 95% confidence limits of haemolysis for the H5 subtype are wider than those for the H11 subtype.

Regional Distribution:

The data derived from the SRH examination of the sera described above were next considered in relational and environmental origin.

(a) Pearl River Delta

The highest occurrence of seropositivity was for the avian H3 subtype (45% to 47%) for which there was no real difference between the urban and rural environments (Table 2). The higher seropositivity for the avian H3 subtype, in contrast to the considerably lower incidences for the other HA subtypes, is probably due to cross-reacting antibodies arising from infection by the Hong Kong variants.

For the other avian HA subtypes, the maximum incidence of seropositivity was 15%. There was evidence of contrasting behaviour between sera from the two environments – urban 0 to 2%, rural 2% to 15% – implying a higher degree of exposure to avian viruses in the rural environment.

(b) Taiwan

Similar finding concerning the H3 subtypes were made although the incidence of human infection appeared a little higher at 97% to 99% (Table 2).

There was also evidence of greater exposure to avian virus in the rural area (urban 0 to 3%, rural 2% to 13%).

Significance of Regional Distribution:

The data in the previous section indicated a higher degree of seropositivity for rural dwellers in both parts of the region this being more apparent for some HA subtypes than others. In order to place this information on regional distribution on a sounder footing the data for comparison were submitted to a statistical evaluation at a 95% con-

fidence level.

When the avian H3 data are excluded from the comparison, there is clearly a higher overall occurrence of seropositivity (incidence of infection) in the rural areas than in the urban localities regardless of the regional area ($p < 0.05$). This difference is perhapes best seen in the comparison of the Pearl River Delta and urban Hong Kong in that the inclusion of the avian H3 seropositivity did not outweigh that of the other avian HA subtypes (Table 2).

The data presented in this study so far show the occurrence of seropositivity to each of the avian and human HA subtypes tested. However, they do not allow interpretation of the occurrence of multiple infection, if any, by these viruses. It is to be expected that people from the urban areas would be exposed to a limited number of viruses and that the occurrence of multiple infections in these two groups would be less than those of the rural groups.

Of the 785 sera examined, excluding data for human and avian H3 subtypes, 238 (30%) showed evidence of infection by one or more avian virus HA subtypes (Table 3). Furthermore, amongst positively reacting sera, some showed haemolysis with as many as four different HA subtypes. Had the results for the avian H3 subtype been included, the number would have risen to five for the rural environment but those data are excluded because of the inability to differentiate a truly avian H3 infection from that due to human Hong Kong variants. Sera from Pearl River Delta and Taiwan showed evidence of multiple infection in 19% and 12% respectively. In contrast, the data from urban Hong Kong and Taiwan produced 2% and 3%, respectively. When data from the rural environments are considered, 21% of the sera show evidence of infection to only one HA subtype of avian virus and 16% of the sera show more than one HA subtype infection. The data showed in table 3 indicates a greater prevalence for multiple infection in the rural as compared to the urban environment.

The data derived from sera from the rural areas showing evidence of multiple infection were further examined to see if a pattern of combinations of antibodies emerged arising from infections by viruses of the different HA subtypes. The three most commonly detected antibodies in the earlier analysis, namely those to the H4, respect to other antibodies. Those to the H4 and H6 virus HA subtypes occurred more often with other antibodies than alone in individual sera whereas antibody to the H11 virus subtype occurred more frequently as a single antibody. This was more obvious in these from Pearl River Delta than those from Taiwan (Table 4).

DISCUSSION

Single raidal haemolysis proved to be suitable test for use in this large scale serological survey for antibodies to avian influenza virus HA subtypes (12). Highlighted th futile efforts of many investigators in the past to detect antibody to avian viruses in serological surveys of animals. (13). It had been generally assumed that avian viruses resided in avian

species without causing antibody response, a notion since corrected by the work of Lu et al (14). and, indeed, the findings of this study. An important extension of this project would be to detect the occurrence of antibodies to avian influenza viruses in domestic poultry in order to see how such information correlated with the result of virus surveillance studies.

Evidence was found of antibody to each of the avian virus HA subtypes examined. However, seropositivity varied considerably in that people from the urban localities possessed antibodies to a narrower range of virus HA subtypes and to a level lower than those from the rural areas. The use of people from Hong Kong Island served as an ideal control for the rural areas and the low level of antibody in these people lends support to the specificity of the SRH test. While it would be reasonable to expect few people on the Island to have been exposed to avian viruses (in contrast to viruses of porcine origin since many pigs are kept on the hillsides).

On the other hand, the higher incidence and range of seropositivity for people from the rural areas could be expected (although had to be proved) because of the greater opportunity for infection arising from the type of agroecosystems of the region. This applied to people in the rural areas of Pearl River Delta and to the farming areas of Taiwan, which are similarly but advanced farming technique than the Delta. The surveillance studies on influenza viruses in the poultry showed a seasonal variation in the occurrence of viruses with influenza isolate predominantly in the hot wet summer months (15). It is possible that the difference in occurrence of antibody between the rural areas of Taiwan and the Pearl River Delta may be merely a reflection of the time of collection of the sera in that those from Taiwan were collected during the cool dry winter whereas those from Pearl River Delta were obtained in June. If antibody to avian influenza virus is only shortlived, then antibodies detected in the sera from the Delta area might be the result of recent infections whereas the Taiwan sera may contain antibodies that have dropped below the level of detection. The slightly higher occurrence of antibody in those sera from urban Taiwan compared to Hong Kong is probably the result of including those persons who had recently migrated to the city from the rural areas.

The most commonly detected antibody was that to the avian H3 virus subtype however, for reasons explained earlier in this study, it is not possible to differentiate between infections due to purely avian viruses because of the high rate of detection of antibodies to the human Hong Kong virus strains.

Antibody to the H4, H6 and H11 virus subtypes were jointly the next most common antibodies detected occurring 9% of the sera. There was, however, a difference in the level of antibody in that the zones of haemolysis for H4 and H6 virus subtypes had mean diameters of 4.5mm whereas those for the H11 subtype had a mean diameter of 4.1mm. If the diameter of the zone of haemolysis is indeed related to the HI titre then the H11 virus subtype has produced antibody to a lower titre.

It was interesting to see that between the two rural populations there was a difference in the occurrence of antibody to particular virus subtypes. In Taiwan, antibody to the

H6 virus subtype was the most common, closely followed by that to H4. In Pearl River Delta, antibody to the H11 virus subtype was the most common, with an occurrence in 15% of the sera. The occurrence of viruses of the H11 subtype in poultry is relatively low. Whether infections by viruses of the H11 subtype result in a more durable immune response in man is a moot point.

On the other hand, infection by viruses of the H5 subtype was lowest. This may simple reflect the lower occurrence of the virus but it is noteworthy that in chickens, Alexander and Parsons (16) showed that prior vaccination with viruses of the H1 (Hswl) subtype provided protection to infection by the H5 subtype. It is, therefore, tempting to speculate that the re-emergence of viruses of the H1 subtype in man may provide protection against H5 infection. There is no evidence of antigenic relatedness of H1 and H5 subtypes.

The findings of this study suggest that human infection by an avian virus would mostly likely take place in the rural areas of the region rather than in the cities. Spresd of virus in man would, however, be rapid in the city as appeared to be the case in the 1968 pandemic. It does seem clear that avian species are the most likely source of virus for man and that the domestic duck is potentially the most significant.

The suggested role of recombination (reassortment) between a human and an animal virus in the emergence of pandemic strains has been reviewed by Scholtissek (17) and will not be considered further. The fact that viruses of a particular subtype are more frequently isloated from ducks does not mean that they alone could be potential pandemic viruses. As this study has clearly shown, antibody was detected to all avian HA subtypes implying that any one of them could be involved. However, before it could do so there would need to be a suitable "ecological niche" for a recombinant virus to establish itself in a group of non-immune susceptibles in the rural areas. It is a matter of speculation how many influenza "brushfire" have occurred in the region but failed to become established. Could the results of this serological study represent such brushfires? A view that may only be resolved well into the next century.

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TABLE 1. OVERALL OCCURRENCE OF ANTIBODY TO AVIAN VIRUS HA SUBTYPES IN 785 SERA EXAMINED BY SINGLE RADIAL HAEMOLYSIS TEST

HA subtype	Number of sera showing haemolysis	Haemolysis zone diameters (mm)		
		Range	Mean	95% confidence limits[d]
H3 (Hav7)	364 (46)%	3.0 – 7.3	4.8	± 0.7
H4 (Hav4)	68 (9)	3.0 – 6.1	4.5	± 0.7
H5 (Hav5)	10 (1)	3.0 – 6.4	4.5	± 1.1
H6 (Hav6)	74 (9)	3.0 – 6.7	4.5	± 0.9
H7 (Hav1)	28 (4)	3.0 – 6.8	4.5	± 0.9
H8 (Hav8)	26 (3)	3.0 – 6.2	4.4	± 0.8
H9 (Hav9)	23 (3)	3.0 – 6.4	4.5	± 0.8
H10 (Hav2)	33 (4)	3.0 – 6.6	4.5	± 0.9
H11 (Hav3)	67 (9)	3.0 – 7.0	4.1	± 0.7
H12 (Hav10)	23 (3)	3.0 – 7.0	4.5	± 0.8
H13	22 (3)	3.0 – 6.5	4.3	± 0.8
H3 (Human)[a]	740 (94)	3.0 – 7.8	5.6	± 0.9

a Human H3 (A/HK/1/68) virus is included for comparison with avian H3 data.

TABLE 2. OVERALL REGION OCCURRENCE OF ANTIBODY TO AVIAN VIRUS HA SUBTYPES AS DETERMINED BY SINGLE RADIAL HAEMOLYSIS TEST

HA subtype	No. of sera positive from		No. of sera positive from	
	Urban Hong Kong (100)	Rural Delta (400)	Urban Tai-chung (95)	Rural Tai-chung (190)
H3	45 (45)%	187 (47)%	40 (42)%	92 (48)%
H4	2 (2)	45 (11)	2 (2)	19 (10)
H5	0	7 (2)	0	3 (2)
H6	1 (1)	46 (12)	3 (3)	24 (13)
H7	0	19 (5)	1 (1)	8 (4)
H8	2 (2)	14 (4)	1 (1)	9 (5)
H9	0	13 (3)	3 (3)	7 (4)
H10	1 (1)	23 (6)	1 (1)	8 (4)
H11	0	58 (15)	2 (2)	7 (4)
H12	1 (1)	13 (3)	2 (2)	7 (4)
H13	2 (2)	12 (3)	2 (2)	6 (3)
H3 (Human)	90 (90)	371 (93)	94 (99)	185 (97)

TABLE 3. OVERALL SINGLE AND MULTIPLE OCCURRENCE OF ANTIBODY TO AVIAN VIRUS HA SUBTYPES

Grouping	Area or locality	Number of* sera positive for any one or more avian subtypes	Number of sera positive for the following number of HA subtypes				Total number of sera positive for	
			One	Two	Three	Four	One HA subtype	Multiple HA subtypes
Urban	Hong Kong (100)	7 (7)	5	2	0	0	16 (8)	5 (3)
	Taichung Taiwan (95)	14 (15)	11	3	0	0		
Rural	Pearl River Delta (400)	152 (38)	78	55	14	5	121 (21)	96 (16)
	Taichung Taiwan (190)	65 (34)	43	14	5	3		
Urban and Rural	All sera (785)	238 (30)	137	74	19	8	137 (17)	101 (13)

* Excluding the avian H3 subtype

TABLE 4. OCCURRENCE OF ANTIBODY TO AVIAN VIRUS HA SUBTYPES IN RURAL AREAS

Rural area	HA subtype	Number of sera showing evidence of		Antibodies most commonly associated in multiple infection
		Single infection	Multiple infection	
Pearl River Delta	H4	14 (4)[b]	31 (8)	H6
	H6	10 (3)	36 (9)	H4
	H11	34 (9)	24 (6)	H4 and H6
Taiwan (190)[a]	H4	7 (4)	12 (6)	H6
	H6	12 (7)	12 (6)	H4
	H11	4 (2)	3 (2)	H6

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血清流行病學在鳥型流行性感冒上之研究

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在人類流行性感冒可能起源於動物之假設下，本實驗利用臺灣、香港及珠江三角洲的城市及鄉村居民之血清標本785件，用單向免疫擴散溶血法，以測定對所有現知鳥型流行性感冒凝血素亞型之抗體效價。

結果顯示，單向免疫溶血測驗，頗適合本研究，在鄉村地區的居民，幾乎對所有凝血素亞型均可測得抗體反應，並且和城市居民之血清陽性反應結果有統計學上之差異（$P<0.05$）。鄉村居民的多重感染率也顯著較高（16 %），尤其是珠江三角洲，因耕耘技術落後，生活環境差，家禽污染率較高，其檢出結果陽性率及多重感染率均最高（38 % 19 %）；而相對的，香港本島居民，因較少和活家禽接觸，故兩者均最低（7%；2%），臺灣居中，可能因城市、鄉村分野不明顯所造成，但鄉村（34 %）和城市居民（15 %）之陽性率仍有明顯的差異（$P<0.05$）。因此，實驗顯示鳥類A型流行性感冒可能會發生動物對人類之感染，而家禽尤其是鴨子泄殖腔對此病毒會產生基因再組合現象，文獻報告最常於鳥類分離出之凝血素亞型為H_4，而本實驗亦顯示鄉村居民H_4之抗體陽性率最高約10 %，是否和流行性感冒病毒變異之未來趨向有關，希望在繼續的努力下，能解答此疑問。

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