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Original Article

Multiannual patterns of influenza A transmission in Chinese live bird market systems

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Background Avian influenza viruses (AIV) cause huge economic losses in poultry industries and pose a substantial threat to human health. However, predicting AIV epizootics and emergence in humans is confounded by insufficient empirical data on the ecology and dynamics of AIV in poultry systems. To address this gap, we quantified incidence patterns for 13 hemagglutinin subtypes of AIV using 6 years of surveillance data that were collected from ten different species of poultry and three different types of poultry holdings (contexts) – retail, wholesale, or farms.

Methods We collected 42 646 samples in Shantou, China between 2000 and 2006. We screened samples for hemagglutinin subtypes 1–13 of AIV and Avian Paramyxovirus-type-1 (APMV-1) using monospecific antisera in hemagglutination inhibition tests. We analyzed the data to determine seasonality patterns, subtype–host, and subtype–subtype interactions as well as subtype bias in incidence in different contexts.

Results H3, H6, H9, and APMV-1 were the most prevalent. No significant seasonality was found when all subtypes were considered together. For most AIV subtypes and APMV-1, there was subtype specificity for host, context, and coinfection partner. H5 showed the most generalized host usage pattern, followed by H9 and H6.

Conclusion Subtype-specific patterns because of host, context, and other subtypes suggest that risk assessments that exclude these details are likely inaccurate. Surveillance should include longitudinal sampling of multiple host species in multiple contexts. Quantitative models of control strategies must consider multiple subtypes, hosts, and source contexts to assess the effectiveness of interventions.

Keywords Avian influenza, coinfection, H5N1, host specificity, live bird market, risk.

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Introduction

Substantial economic losses to poultry industries are caused by both low- and high-pathogenicity subtypes of influenza A.[1–3] High levels of replication and transmission of the viruses in domestic poultry can also lead to mutation or reassortment that generates virulent novel strains which can spillover to humans.[4–10] Live bird markets provide prime conditions for the generation of novel subtypes because they are mixing grounds for different poultry species infected with different subtypes, infection rates are high, and there is a constant inflow of infected and naïve hosts.[11–14] Movement of humans between farms and markets can exacerbate this further.[13,15] The ability to predict outbreaks of Avian influenza viruses (AIV) in poultry operations is crucial for minimizing economic losses and for preventing spillover to humans. However, the anticipation of outbreaks requires an understanding of how the interactions between multiple subtypes and hosts impact the epidemiological dynamics of AIV's. At present, empirical data on host specificity, seasonal variation, and subtype co-occurrence in locations where poultry species mix are lacking.

High pathogenic avian influenza (HPAI) H5N1 has devastated poultry operations and caused human deaths, in part because of a decreased barrier to interspecies transmission, which evolved during transmission in domestic poultry.[8] In this respect, we expect that H5N1 viruses would show less discrimination between the poultry species they infect. Although surveillance in farm-to-market systems is

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widespread,[16–23] to date, no predictive surveillance tools for AIV have been reported. This gap is because of a lack of intensive longitudinal sampling within a given location, including data from multiple hosts and subtypes (which together drive subtype-specific dynamics). Here, we describe analyses of samples taken from live bird markets (retail and wholesale) and farms in a single city in southern China over a 6-year time period. We used these data to identify subtype-specific patterns of host association, coinfection, and seasonality and thus present an empirical foundation for constructing predictive tools of AIV dynamics and evaluative tools for control in southern Chinese live bird markets.

Materials and methods

Data collection

Routine sampling of bird species in nine live bird markets was conducted in the city of Shantou, China. Sampling was conducted at 2–4-week interval with reliable frequency between October 2000 and October 2006. Data were aggregated on a monthly scale. Only three of the host species were sampled consistently throughout the entire time period: *Anas platyrhynchos* (duck, domestic, and wild), *Coturnix japonica* (Japanese quail), and *Gallus gallus* (domestic chicken and silkie chicken) (Figure S1); and thus, analyses on the full time series were limited to these species. *Phasianus colchicus* (pheasant), *Alectoris chukar* (chukar), *Numida meleagris* (guinea fowl), and *Columba livia* (pigeon) were sampled consistently during the last 3 years, whereas *Francolinus pintadeanus* (partridge) were sampled during the first 4 years (Figure S1). *Meleagris gallopavo* (turkey) and *Anser anser* (domestic goose) were only sampled during a 1-year period. Although other host species were sampled sporadically, we excluded these data because their sampling times were inconsistent (because of their presence in markets) and sample sizes were small (<80). These were as follows: *Anas crecca* (teal), *Pycnonotus sinensis* (Chinese bulbul), *Acridotheres tristis* (starling), and some unidentified species of shorebirds and parrots. Between May 2005 and October 2006, surveillance in retail markets was expanded to nearby farms and wholesale markets where samples from *An. platyrhynchos, Ar. anser,* and *G. gallus* (wholesale markets), or *An. platyrhynchos* and *G. gallus* (farms) were collected (Figure S2). We refer to the three types of poultry holdings as ''contexts.'' The proportion of samples from each context was as follows: retail markets (n = 27 331; 64%), wholesale markets (n = 5420; 13%), and farms (n = 9925; 23%). Table 1 summarizes the sampling effort and infection rates by host summed across contexts. In the Shantou wholesale markets, live birds (especially chickens) come from all farm sectors. This includes integrated enterprises and medium sized poultry industries where birds are kept only indoors and separated from other avian species (sector 1 and 2 by the FAO definition) as well as poultry from small farms and private owners (sectors 3 and 4). Our farm surveillance system in Shantou mainly covered sectors 2–4, with a smaller proportion from sector 1.

Virus was isolated from samples using embryonated chicken eggs. AIV subtypes H1-13 and Avian Paramyxovirus-type-1 (APMV-1) were identified using monospecific antisera in hemagglutination inhibition (HI) tests.[24] We did not distinguish between subtypes of APMV; our screening method only identified whether APMV-1 was present. All 13 hemagglutinin subtypes of AIV were observed at least once. In initial screens, we used the WHO reference subtyping antisera to identify virus subtypes, but we continually updated our antisera based on our most current field data as follows. Representative viruses with lower hemagglutinin inhibition levels were genetically analyzed. If we found that the phylogenetic relationship between current isolates and the WHO reference viruses was divergent, we used the newly isolated strains to make new subtyping antisera. Only those strains showing broader reactivity to different antigenic groups within a specific subtype (but no cross-reactivity to other subtypes of the virus) were used. For some subtypes, multiple antisera were used for diagnosis. For example, we used two antisera to detect and distinguish viruses from both G1 and Y280/Ck-Bei lineages. We classified samples as multi-subtype infections when the virus isolates from a single sample showed an HI titer of 40 or greater against reference antisera of more than one subtype.

Seasonality

To evaluate whether there were any regular cycles (seasonality) in incidence, autocorrelation coefficients between incidence at time t and $t + i$ (where i = 0,1,...24) were calculated for all subtypes together as well as for each subtype with enough data to be considered alone, and for APMV-1. Cross-correlation between dominant and minor subtypes of AIV and APMV-1 was conducted by Pearson correlation analysis for lags 0–24 to evaluate whether dominant subtypes tended to be in phase with each other or other subtypes. Analyses were limited to specific hosts (to distinguish real seasonality from host effects), which were sampled most intensively and consistently during the entire time series (ducks, chickens, and quail).

Host usage and associations with poultry context

Subtypes with high enough prevalence for these statistical analyses were as follows: H1, H3, H4, H5 (mostly HPAI H5N1), H6, H9, H11, and APMV-1. The host association analysis was conducted for the retail market time series using the last 3 years of data (October 2003–September

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Table 1. Summary of sampling and rates of infection and coinfection in all hosts and all contexts.

Common name	Scientific name	Abbreviations	*n* Samples	*n* Positives	Infection Rate (%)*	% of Positives**	*n* Co-infections	Co-infection Rate (%)***	% of co-Infections†
Waterfowl	Anseriformes								
Domestic duck	*Anas platyrhynchos (domesticus)*	DK	15657	2805	17.9	49.3	481	17.1	73.9
Wild duck (mallard)	*An. platyrhynchos*	WDK	2822	337	11.9	5.9	58	17.2	8.9
Domestic goose	*Anser anser (domesticus)*	GS	7025	241	3.4	4.2	16	6.6	2.5
Land-based birds									
	Galliformes								
Chicken	*Gallus gallus (domesticus)*	CK	7570	708	9.4	12.4	31	4.4	4.8
Silkie chicken	*Gallus gallus (domesticus)*	SCK	1917	282	14.7	5.0	19	6.7	2.9
Japanese quail	*Coturnix japonica*	QA	3022	619	20.5	10.9	25	4.0	3.8
Partridge	*Francolinus pintadeanus*	PA	744	203	27.3	3.6	10	4.9	1.5
Chukar partridge	*Alectoris chukar*	CU	1167	224	19.2	3.9	6	2.7	0.9
Common pheasant	*Phasianus colchicus*	PH	1125	169	15.0	3.0	2	1.2	0.3
Domestic turkey	*Meleagris gallopavo*	TK	84	8	9.5	0.1	0	0.0	0.0
Domestic Guinea fowl	*Numida meleagris*	GF	323	51	15.8	0.9	2	3.9	0.3
	Columbiformes								
Pigeon	*Columba livia*	PG	1190	42	3.5	0.7	1	2.4	0.2
		Totals	42646	5689	13.3	100	651	11.4	100

*% of samples for host *i*.
**% of all positive samples.
***% of positive samples for host *i*.
†% of all co-infections.

2006; the longest section of data with the highest number of host species sampled consistently). We constructed an expected distribution of virus prevalence for each subtype-by-host combination under the null hypothesis that virus subtypes infected hosts from each group with equal probability. Thus, the expected prevalence for a subtype in a given host group in a given month was equal to the proportion of samples of that host type times the total prevalence in all hosts in that month. If the difference between observed and expected values for a given subtype on a given host was zero, then there was no bias for that host type. Thus, if a given subtype infects host groups at random, the distribution of differences (observed-expected) should be the same across all host groups with a median value that is not significantly different from 0. We tested this hypothesis for each virus subtype using non-parametric Kruskal–Wallis tests, excluding time points with no infections in one or more host groups. Because the null hypothesis of no host bias was rejected in all cases, we conducted sign-rank tests for the data from each host group (applying a Bonferroni correction to significance levels for each data set; $\alpha = 0{\cdot}05/7 = 0{\cdot}0071$) to examine in which hosts the subtype was over- or underrepresented. Median values below 0 indicated underrepresentation, whereas those above 0 indicated overrepresentation. The context preference analysis was carried out similarly except that the data were divided by contexts (retail market, wholesale market, farms) and limited to either ducks or chickens (because of sample limitations, Figure S2) to exclude host effects. Only 1·5 years of data (June 2005–September 2006) were available for the context preference analysis.

Subtype–subtype associations

We examined whether subtypes coinfected with other specific subtypes more often than by chance, using a multinomial test with only the double infection data (we excluded higher order infections because these data were rare and difficult to interpret). This analysis was limited to ducks to dissect subtype associations from host associations. It was necessary to include data from all contexts in order to have large enough sample size. The null hypothesis for this test was that subtype *i* would coinfect with any other subtype *j*

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with equal probability such that the expected counts for partners of subtype i would be proportional to the frequency of each partner in all single (s) and double (d) infections. Thus, the expected counts for each j partner of subtype i, $E[X_j]$ are:

$$E[X_j] = \left(X_j^{s+d}/N_j^{s+d}\right) * X_i^{d},$$

where $X^{s+d}{}_j$ is the total number of single and double infections for partner j, $N^{s+d}{}_j$ is the total number of single and double infections for all j partners, and $X^{d}{}_i$ is the total number of double infections for subtype i. The maximum likelihood estimates of the parameters for the null multinomial model for each subtype i are then: $\pi_j = \mathrm{E}[X_1]/\sum(E[X_j])$, $E[X_2]/\sum(E[X_j])$,..., $E[X_n]/\sum(E[X_j])$, where $i = 1, 2, ..., n$ focal subtypes. Likewise, the maximum likelihood estimates for the parameters given the data are $p_j = X_1/\sum(X_j)$, $X_2/\sum(X_j)$,..., $X_n/\sum(X_j)$. The probabilities under the null and alternative models are:

$$P(X)_0 = N_j^{d}!\Pi(\pi_j^{xi}/X_i!) \text{and} P(X)_A = N_j^{d}!\Pi(p_j^{xi}/X_i!)$$

and the likelihood ratio test statistic D is $-2\ln(P(X)_0/P(X)_A)$, which is approximately distributed χ^2_{n-1}. The likelihood ratio statistic was divided by the correction factor $1 + \sum\left(\pi_j^{-1} - 1\right)/6N_j^{d}(n-1)$ to decrease type I error inflation because of a difference between the moments of the likelihood ratio statistic and the chi-square distribution.[1]

Results

Temporal trends

Prevalence varied substantially with APMV-1 and AIV subtypes H3, H6, and H9 being the most prevalent (Figure 1A,B). Influenza subtype H6 reached as high as 20% prevalence among all poultry species, while other subtypes, including H5, rarely reached 5%. Although quail consistently showed the highest infection rates (Figure 1C), no significant differences were observed in the mean prevalence across the three most intensively sampled host species when considering all points in the time series (Figure 1C, Table 1).

To identify whether there were significant temporal patterns in Figure 1A, we conducted autocorrelation analyses for all infections together versus each subtype individually. We did these analyses in separate host species to disentangle regular patterns of incidence from effects because of host species composition. In ducks and chickens, there was no significant seasonality when all subtypes were considered together, whereas there was significant annual

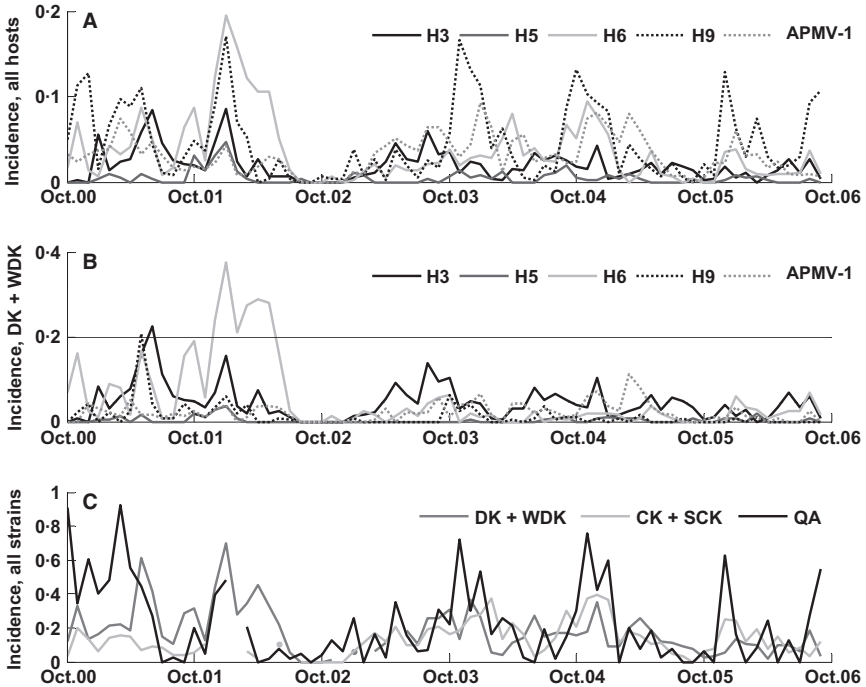

Figure 1. Temporal trends in retail markets. (A) shows the monthly proportions for all host samples positive for: APMV-1 (gray, dotted); the most prevalent Avian influenza viruses (AIV) subtypes (H3, black; H6, light gray; H9, black dotted); and H5 (dark gray). B shows the same analysis limited to ducks (*An. platyrhynchos*). The horizontal line marks the upper limit in plot A for comparison. C shows monthly proportion of samples positive for subtypes H1-H13 or APMV-1 for the three host species (as in Table 1) that were sampled consistently throughout the 6 years (see Figure S1 for the time series of sample sizes).

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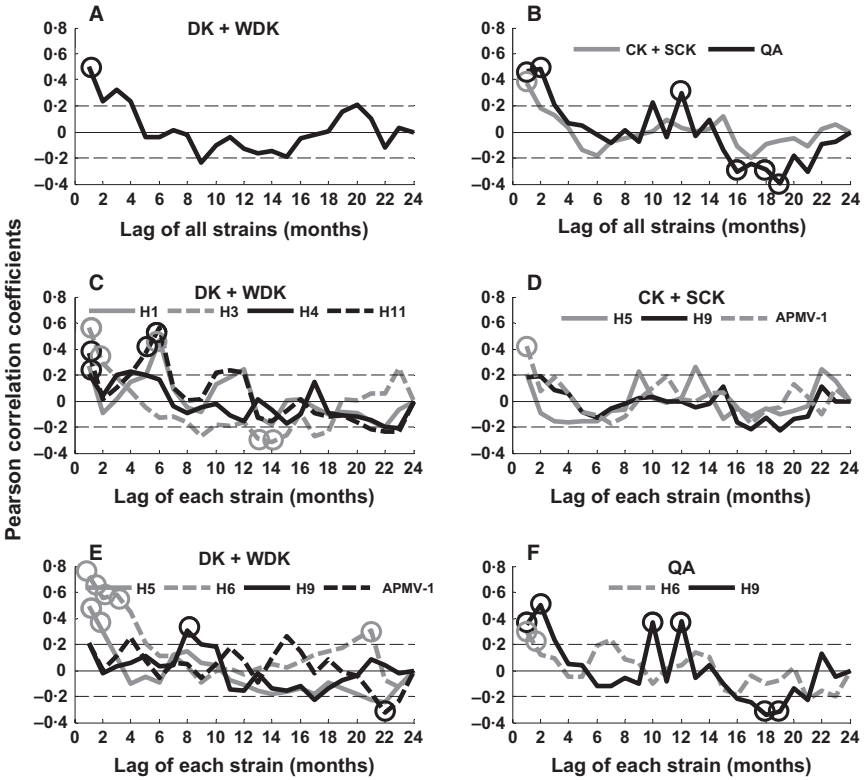

Figure 2. Autocorrelation functions for all subtypes together and separately in different hosts. The Pearson correlation coefficients for data lagged by each number on the *x*-axis are plotted for subtypes isolated from ducks (DK + WDK; A, C, and E), chickens (CK + SCK; B and D), or quail (QA; B or F). A and B show correlations when all subtypes are aggregated, whereas C-F show correlations for individual subtypes. Circles indicate significance of the autocorrelation when data are lagged by *x*-axis values ($\alpha = 0.05$). Values above 0 indicate positive correlation (i.e., the incidence at times *i* and *j* are either both high or both low), whereas values below 0 indicate negative correlations (i.e., the incidence at times *i* and *j* are opposite– one is high and one is low).

seasonality in quail (Figure 2A,B). However, when considering the subtypes individually in ducks, H11 showed biannual patterns, H9 peaked at 8-month intervals, and H3 showed crashes at roughly 1-year lags and a trend of biennial peaks (Figure 2C,E). H6 showed significant positive autocorrelation at lags 1–4 and a roughly biennial pattern suggesting that it is present relatively consistently with larger peaks every 2 years (Figures 1B and 2E). H5 showed no evidence of temporal correlations other than at lags 1–2. In contrast, in chickens, none of the subtypes (H5, H9, or PMV – the viruses that were most common in chickens) showed temporal patterns individually. In quail, H9 showed strong annual seasonality, whereas H6 showed no specific pattern.

We also investigated cross-correlations between each of the dominant subtypes (H3, H6, and H9) and other viruses with sufficiently high prevalence (H1, H4, H5, H11, and APMV-1) in ducks to determine which subtypes showed similar temporal patterns (data not shown, α for Pearson correlation was 0·05). H5 and H9 were positively correlated with H3 in the same month, whereas H4, H9, and APMV-1 were negatively correlated with H3 at 12–15-month intervals. H6 was positively correlated with H9 in the same month, and H5 was also strongly positively correlated with H9 at 8-month intervals, which is the regular temporal pattern for H9 that was indicated in the autocorrelation analysis (Figure 2E). In contrast, H4 was negatively correlated with H9 at a similar lag (9 months). Other than H5, none of the minor subtypes or APMV-1 showed correlation with the dominant subtypes H6 and H9 in the same month. However, many of the subtypes showed negative correlations with H6 between lags 4 and 12 months, again, highlighting the non-specific incidence patterns of H6.

Host and context biases

H1, H3, H4, and H11 were overrepresented in ducks and underrepresented in all other host species, whereas H5, H6, H9, and APMV-1 showed different, more inclusive host usage patterns (Figure 3A). H5 showed the most non-specific host usage pattern with its random association to all hosts except for an underrepresentation in chukars and pigeons (i.e., where random association means that the subtype infects host species *X* in proportion with the number of samples from host species *X*). H6 was randomly associated with ducks and quail, overrepresented in

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A

	DK + WDK	QA	CK + SCK	PH	CU	PG	GF	Positives
H1	87, 26	1, 26	2, 26	0, 26	0, 26	0, 26	0, 19	90
H3	204, 35	2, 35	4, 35	0, 35	2, 35	0, 35	1, 27	213
H4	89, 29	0, 29	0, 29	0, 29	1, 29	0, 29	0, 23	90
H5	18, 23	4, 23	13, 23	13, 23	3, 23	1, 23	2, 17	54
H6	81, 32	110, 32	2, 32	18, 32	149, 32	0, 32	15, 32	375
H9	43, 35	202, 35	251, 35	118, 35	39, 35	1, 35	16, 35	670
H11	74, 21	0, 21	1, 21	0, 21	0, 21	0, 21	0, 16	75
APMV-1	146, 35	3, 35	261, 35	14, 35	10, 35	31, 25	12, 35	477
Samples:	5432	1513	3070	887	981	960	261	

B DK

	Farm →	Wholesale →	Retail	Positives
H1	83, 13	75, 13	11, 13	169
H3	146, 18	261, 18	60, 18	467
H4	65, 18	4, 18	18, 18	87
H5	6, 3	0, 3	4, 3	10
H6	452, 18	524, 18	40, 18	1016
H9	41, 14	29, 14	3, 14	73
H11	61, 12	62, 12	8, 12	131
APMV-1	52, 15	2, 15	19, 15	73
Samples:	5041	1806	1681	

C CK

	Wholesale	→ Retail	Positives
H1	1,1	0,1	1
H3	1,1	0,1	1
H4	1,1	0,1	1
H5	11,2	1, 2	12
H6	2, 2	1, 2	3
H9	39, 15	58, 16	97
H11	NaN	NaN	0
APMV-1	12, 14	41, 15	53
Samples:	1691	849	

Figure 3. Patterns of host usage and context dependence. A shows host usage for host species that were sampled consistently over the longest time period. Only the last 3 years of retail market data (September 2003–September 2006) were included because this was the time period during which the most host species were sampled consistently (see Figure S1) and surveillance protocols were unchanged. Context dependence patterns for subtypes are shown in ducks (B) and chickens (C). Farms were excluded in C because chickens were not sampled intensely enough (see Figure S2). Gray boxes indicate a significantly positive relationship, black boxes are for significantly negative relationships, and white boxes indicate that the infection rate is proportional to the number of samples collected. Numbers inside the boxes: # of positive samples, number of monthly time points in analysis. Only subtypes for which there were adequate samples, and host species that were sampled consistently, were included in the analysis. Hosts are listed across the top (abbreviations as in Table 1). Subtypes are listed in the first column; Avian Paramyxovirus-type-1 is Avian Paramyxovirus-type-1. The total number of samples collected from each host species is listed along the bottom. The total number of positive samples for each subtype is in the last column. For each subtype, we excluded time points in which no positive samples were found (reflected in the second number in each box). Bird groups with multiple subspecies from the same species were pooled in A (a preliminary analysis showed that there were no differences between these groups). Alpha values were adjusted for multiple tests using a Bonferroni correction ($\alpha_A = 0.0071$, $\alpha_B = 0.0167$, $\alpha_C = 0.025$).

chukars, and underrepresented in chickens, pheasants, and pigeons. H9 was overrepresented in quail and chickens, underrepresented in ducks, chukars, and pigeons, and randomly associated with pheasants and guinea fowl. APMV-1 was overrepresented in chickens and underrepresented all other hosts except pigeons and guinea fowl where it was randomly associated. Although we were unable to include partridge in the host usage analysis (because of a difference in sampling time relative to other hosts), the infection results are worth noting because infection rates in partridge were the highest for any host species at 27·3% (Table 1). Almost all of the infections in partridge included H5 (6·4%), H6 (17·7%), H9 (73·5%), and APMV-1 (8·4%) (Table S1). Similarly, these four subtypes caused most of the infections in other minor poultry species: pheasant, chukar, partridge, and guinea fowl (Table S1). H5 was most prevalent and caused the highest number of infections of any subtype, in domestic geese.

The host association patterns for the most frequent subtypes of AIV (H3, H6 and H9) and H5 were generally consistent in time with a few notable exceptions (Figure S3). H5 and H6 show a trend of increased affinity for ducks through time, and H6 shows a decreasing affinity for chukars. These trends could explain why these subtypes show random association with these hosts. Alternatively, temporal changes in host association (that do not match seasonal host species abundances because of holiday festivities) could indicate that newly evolved genotypes have emerged, with an altered host usage pattern.

In ducks, four of the eight subtypes also showed context-dependent patterns (Figure 3B,C). H3 and H6 were consistently overrepresented in wholesale markets, whereas H4 and APMV-1 were underrepresented (Figure 3B). H6 was also underrepresented in retail markets, while H3 and H4 were present in retail markets at levels that would be expected based on overall prevalence and sample sizes from each location. When conducting the same analysis in chickens, APMV-1 was underrepresented in wholesale markets and overrepresented in retail markets (Figure 3C). H9 showed no context-dependent incidence patterns in either ducks or chickens. These patterns were generally consistent in time except that H3 showed a trend toward increased prevalence in farms and decreased prevalence in retail markets with time (Figure S4).

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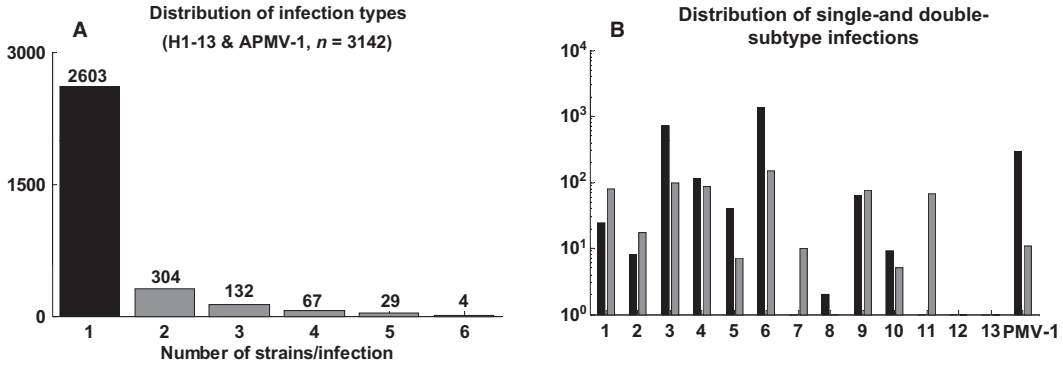
Detection of multiple subtypes per sample

In the full data, 651 of 5689 (11·4%) positive samples generated positive HI results for more than one subtype (Table 1). We interpreted these multiple positive results as coinfections and not cross-reactivity, because reference antisera for each subtype were updated a number of times to track antigenic changes (see Methods), and no temporal association in patterns of multiple positive results was detected (Figure S5). Ducks accounted for most of the coinfections (82·8%; 539/651) and had a coinfection rate of 17·2% [539 of 3142 positive duck samples contained more than one subtype (Figure 4A)]. Most of the multi-subtype samples were double infections, but higher order infections were also observed consistently (Figures 4A, S5), demonstrating the widespread regular occurrence of multi-subtype infections in ducks. When comparing the frequency of coinfection in ducks across contexts, farms showed higher levels of coinfection (21·6%) relative to wholesale markets (14·8%) and retail markets (16·1%) (data not shown). Owing to the strong patterns of host specificity (above), we limited all other coinfection analyses to duck samples, where an overwhelming majority of multi-subtype samples were found.

There was a difference among subtypes in the likelihood of being detected in a coinfection (Figure 4B). When considering single and double infections only, rarer subtypes such as H1, H2, H7, H10, H11, and H12 were detected in double positive samples more often than in single positive samples, while the common subtypes, H3, H6, H9, and APMV-1, as well as H4 and H5, were mainly detected as single positive samples. One exception is that H9 was detected in single versus double infections at similar levels in ducks (Figure 4B), but was much more frequent in single relative to double infection when all hosts were



C

	H1	H3	H4	H5	H6	H7	H9	H11	APMV-1	*P*	% co
H1	**24**	1	1	2	5	0	0	66	2	<0·0001	76·2
H3	1	**702**	32	1	30	4	8	0	2	<0·0001	10·0
H4	1	32	**118**	2	50	0	0	0	2	<0·0001	42·4
H5	2	1	2	**40**	1	0	0	0	0	ns	13·0
H6	5	30	50	1	**1345**	2	61	0	2	<0·0001	10·1
H7	0	4	0	0	2	**0**	4	0	0	0·053	100·0
H9	0	8	0	0	61	4	**65**	0	2	<0·0001	53·6
H11	66	0	0	0	0	0	0	**0**	0	<0·0001	100·0
APMV-1	2	2	2	0	2	0	2	0	**290**	ns	3·3

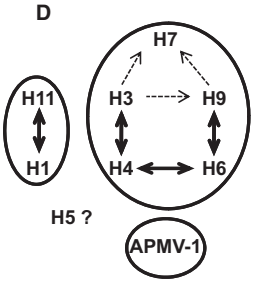


Figure 4. Patterns of multi-subtype infections in ducks in all contexts. Part A shows the distribution of numbers of subtypes per sample. For example, the first bar (black) indicates that there were 2603 single-subtype infections in ducks. *N* is the total number of positive samples. The inset shows the distribution restricted to multi-subtype infections only (no single positive samples). Part B shows counts of single- (black) and double-subtype (gray) positives for each subtype (shown on *x*-axis; numbers are Avian influenza viruses (AIV) subtypes 1–13). Counts of double positives are the total number that include each subtype (i.e., the sum of gray bars is greater than the total number of double infections). H12 and H13 are blank because they were only observed in infections with 3 or more subtypes. Part C shows partner bias in double infections. Rows are the focal subtypes and columns are their partners. Numbers in the boxes are the count of double infections with each partner; single infection counts are shown in the diagonal. Gray indicates subtype coinfections that occur more often than expected based on prevalence, whereas black indicates coinfections that occur less often than expected. Expected count for a given partner was based on its frequency in single and double infections, and significance was tested by a multinomial test (see Methods and Supporting information). Uncolored blocks represent combinations with either no significant bias in subtype coinfections or very weak trends. The last column indicates the percent of infections that are coinfections for focal subtypes. Part D shows a schematic relationship of coinfection compatibility (double infections only). Subtypes connected by a double arrow have significant associations with each other, whereas unconnected subtypes are found together less often than expected based on prevalence. One-sided arrows indicate the direction of one-sided associations. Circles indicate distinct phenotypic clusters of compatibility; that is, Avian Paramyxovirus-type-1 (APMV-1) shows no association with AIV for coinfection and most subtypes of AIV show significant avoidance of APMV-1. There is not enough sample size to classify H5.

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considered (data not shown). As H9 is relatively rare in ducks compared to its frequency in other hosts, this is consistent with the above result that rarer subtypes tended to be found in coinfections at higher frequency than more common subtypes.

Lastly, we examined whether subtypes showed a bias in the subtypes with which they coinfected, again, limiting the analysis to ducks. The following significant reciprocal subtype associations were found: H1-H11, H3-H4, H4-H6, and H6-H9 (Figure 4C,D). The coinfection patterns highlight clusters of subtypes wherein reassortment is more likely and are consistent with the finding that reassortment of H6 and H9 is more common than with other subtypes.[14,25] A full matrix of coinfection occurrences, including rarer subtypes and coinfections with >2 subtypes, is given in Table S2.

In general, H3 was less likely to be found in a coinfection with the other frequently detected subtypes H6 and H9 (Figure 4C). The rarer subtypes H1 and H11 showed a strong association only with each other, although H1 coinfected with a few other subtypes (notably H6 and H5). H4 also showed strong association with only a few subtypes, H3 and H6, but did coinfect with H5 on occasion. H3 and H9 showed some association with H7. Although sample sizes within a single host species were too small to draw conclusions about coinfection patterns of H5, examination across host species showed that H5 shared 19 double-strain infections, mainly with H6 and H9, but also with H1, H3, H4, and APMV-1 (Table S3). APMV-1 showed no association with any subtype of AIV and most subtypes tended not to coinfect with APMV-1.

Discussion

This study examines the dynamics of 13 hemagglutinin subtypes of AIV as well as APMV-1 in southern Chinese poultry systems and identifies subtype–host and subtype–subtype associations. Most subtypes showed significant patterns of host specificity, context preference, and discrimination between coinfection partners. H5, H6, and H9 showed the most generalized host usage pattern, H6 and H3 showed the widest range of double infection partners in ducks, and H1 and H9 also showed a wide range of coinfection partners when higher order infections were considered.

This previously undemonstrated promiscuity of H5N1 (although hypothesized through genetic analyses) is proposed to have originated by an early reassortment event followed by transmission through the mixed poultry populations in farms and markets in China, which resulted in selection of HPAI H5N1 viruses that are adapted to multiple hosts and have reduced barriers to interspecies transmission.[8] The generalized host usage and coinfection pattern of the human-infectious subtypes, H5 and H9 (mostly H5N1 and H9N2), has potentially important consequences for the evolution of each other and other dominant subtypes such as H3 and H6. As we and others[25,26] have found, H6 and H9 are predominant in Asian markets, and reassortments of them with each other and H5 have been identified.[27–29] Our findings suggest that the potential for reassortment is related to subtype-specific host usage patterns and coinfection probability. Surveillance for several subtypes, especially H6 and H9, should be emphasized in addition to H5 surveillance in order to appropriately assess emergence of a pandemic strain.[14,30,31]

The high infection rates and overrepresentation of H9 in quail support experimental studies and sequence data suggesting that quail could be a key intermediate host for adaptation of H9 from wild birds to domestic poultry.[9,32,33] Fewer genetic changes are needed in H9 viruses for efficient replication in quail relative to chickens.[34] There is, therefore, a potential facilitator for emergence of H9 in humans because H9 subtypes are dominant in retail markets, have generalized host usage pattern, are capable of establishing coinfection with other subtypes (H3, H5, H6, H7, and APMV-1), and are known to cause human infections [35–40] (and our unpublished data showing that 0·65% of 4700 human samples were seropositive for H9N2).

In southern China, four avian influenza virus lineages, HPAI H5N1, W312-like H6N1, G1-like H9N2, and CK/bei-like H9N2, became established in land-based poultry (especially birds from the superfamily Phasianoidea, Order Galliformes; CK, PA, QA, and CU) in 1997 and are now endemic.[14,25,27,41] Moreover, all four lineages were prevalent in our study area during the study period, which partly explains the high infection rates in the minor poultry species (e.g., partridge with 27·3% infected). Another potential reason for the particularly high infection rate in partridge is that partridge are predominantly raised in small farms, backyards, or villages, where birds are kept outdoors with access to other species of poultry or even wild birds. Also, as vaccination is uncommon for partridge (and other small, minor poultry such as chukar and quail), these birds may have less immune protection against the endemic strains. However, as experimental studies have not yet examined potential mechanisms for this remarkably high susceptibility of partridge, it is difficult to determine whether their high infection rates are indeed because of the conditions in which this poultry species is managed or whether there is some special genetic basis.

There was no significant seasonality in ducks or chickens when all subtypes were considered together, but significant temporal patterns emerged when subtypes were analyzed separately. In ducks, a range of subtype-specific temporal patterns emerged, ranging from biannual to biennial cycles. One exception was H6 that showed erratic temporal behavior. None of the most frequent subtypes in chickens

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(H5, H9, and APMV-1) showed temporal patterns, whereas there was a strong annual pattern for H9 in quail. The wide range of subtype-specific patterns is different from those that have been observed in some migratory bird populations (high in late summer, early fall) [42,43] and an analysis of short-term longitudinal AIV surveillance data in domestic poultry operations in South–East and East Asia (annual cycles with peaks in winter).[44] In the latter study, much of the data were cross-sectional, and sampling during the summer months was sparse. The additional patterns identified in our data, showing that seasonal peaks are not restricted to a particular time of year, and do not happen in concert for all subtypes or host types, highlight the complexity of the ecological drivers in these types of environments. To mechanistically understand these epidemiological drivers in live bird markets, more studies of long-term continuous monitoring of multiple subtypes in multiple host species, which include the host population dynamics, are important.

The difference between the annual seasonal patterns of AIV prevalence for all subtypes in migratory birds and the subtype-dependent temporal patterns in domestic poultry is likely due to the difference between these two environments in bird population dynamics. Wild bird populations contain all age groups, seasonal fluctuations in population density, and a seasonal birth pulse of naïve host input, whereas bird populations in live bird markets are more homogenous, consisting of a continuous flow of young, naïve hosts, and a relatively constant host density. However, seasonality could exist in contrived environments such as live bird markets because host distributions could shift at regular intervals because of market demands, and interventions, when used, are typically employed with some temporal pattern. For example, it has been shown that hygiene protocols used at similar live bird markets in Hong Kong caused temporary decreased prevalence followed by a recovery in infection rates,[45] which could appear as regular cycles of infection. A second reason for the difference in temporal patterns between wild and poultry populations could be vaccination (especially the use of vaccines with improperly inactivated strains which is known to occur in both large-scale and small poultry operations in this region). The use of a prevailing strain in vaccines may result in subtype replacement (antigenic shift) and subsequent reemergence of a novel strain (antigenic drift),[46–48] which could manifest as regular temporal changes in subtype prevalence. Hence, a mechanistic understanding of the unique ecology (bird demographics, changes in species composition, intervention routines, and prevalence of multiple subtypes) of a live bird market is crucial for disease risk prediction within that market.

The complex patterns of host and context specificity described here emphasize that the dynamics of AIV in poultry systems cannot be understood from models or data that only consider a single host type in a single context. Changes in host specificity may indicate adaptation and hence an increased risk of emergence of a novel strain. Although we predicted that subtypes would show host specificity, the non-random association of subtypes with context was unexpected and raises hypotheses that should be tested. The context-dependent patterns could be driven by either subtype life-history characteristics (i.e., incubation or infectious period that favors the time spent in a specific context) or different host population structure within different types of farms, wholesale, and retail markets. Distinguishing between these hypotheses is important for constructing analytical tools that evaluate control measures, for implementing cost-effective controls within each context and for ultimately constructing poultry systems that minimize the risk of emergence.

We observed a high level of multiple-subtype infections: 11·4% (all hosts), most of which were double infections. Overall, ducks showed the highest rate of coinfection (17·2%), which likely reflects the fact that they are primary hosts to all influenza subtypes.[42] In these birds, AIV mainly replicates in the intestine[49]; thus, the high rates of coinfection could be due to consuming water from shared sources (which is known to be a source of infection in live bird markets[50]). The higher coinfection rates of ducks in farms may be due to the fact that they are housed together in this context whereas there is relatively less opportunity for duck–duck interaction in retail markets. It is also noteworthy that the most prevalent subtypes (H3, H6, and H9) and APMV-1 were mainly observed in single infections while rarer subtypes were observed in multi-subtype infections more often than in single infections. These findings are consistent with a study in wild birds,[51] which suggested that rarer subtypes persist through interactions with more common subtypes. These studies and our data support that studies should be designed to test possible mechanisms explaining the persistence of rare subtypes through interactions with other subtypes.

Our data were collected as part of a routine surveillance program, not designed specifically for the questions we addressed in this study. Consequently, our methods for identifying coinfection patterns have some caveats that should be taken into account when interpreting our results. As with any serological test, antibody cross-reactivity can confound results. However, antigenic recognition was confirmed regularly and always after a change in antisera, which was implemented after suspected antigenic shifts. Also, spot checking for some of these samples by 454 sequencing revealed a false positive rate of 3% because of cross-reactivity, which is very low. Thus, we do not feel that this contributes systematic bias to our results. Secondly, viruses were initially isolated in eggs prior to

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subtyping, which could select against subtypes that replicate poorly in eggs in the presence of a coinfecting strain. This potential source of bias should not affect results of positive associations between subtypes but could explain some of the negative associations. Recently, we used 454 sequencing on some of the original swabs and found coinfections that had not been identified by virus culture. Thus, studies that are specifically designed to test for subtype–subtype associations are needed to get the most accurate picture of subtype interactions.

To make predictions about the emergence risk of novel strains of influenza A, we need surveillance and models based on subtype dynamics in several different live bird markets and their source contexts. We presented a unique multiannual time series that shows that AIV prevalence depends on multi-host and multi-subtype data and that interactions change over time. Our study demonstrates that these interactions should not be excluded from analytical tools developed to evaluate methods of control in live bird markets. To gain a deeper understanding of how the complex ecology of live bird markets affects AIV incidence, it is crucial that more studies such as ours are conducted in other live bird markets and that host population dynamics are also recorded.

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Addendum

KMP conceived the analyses, analyzed the data, and wrote the manuscript; SR conceived the analyses, gave advice on analyses, and wrote the manuscript; JW, WH, HZ, and YG designed and collected the data and edited the manuscript; CTW, GJDS, MP, and PH participated in conceiving the analyses and edited the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Infections in each host species by subtype.

Table S2. Co-infection matrix for each strain in ducks.

Table S3. Co-infections for H5 in different host species.

Figure S1. Host sampling in retail markets.

Figure S2. Host sampling in farms and wholesale markets.

Figure S3. Temporal patterns of host usage for the most prevalent strains and H5 in 5 hosts.

Figure S4. Temporal patterns of occurrence in different contexts for the most prevalent strains and H5.

Figure S5. Time series of the proportion of al l infections containing × number of strains for different sets of hosts and contexts.

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