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Risk assessment of 2024 cattle H5N1 using age-stratified serosurveillance data

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ABSTRACT

The highly pathogenic avian influenza virus A(H5N1) clade 2.3.4.4b has caused a human outbreak in North America since March 2024. Here, we conducted a serosurveillance study to determine the risk of A(H5N1) clade 2.3.4.4b (2024 cattle H5N1) to general population. In the initial screening of 180 serum specimens encompassing all age groups, 2.2% (4/180) had detectable neutralizing antibody (nAb) titres against reverse genetics-derived 2024 cattle H5N1, with all collected from older adults aged ≥ 60 years old. Further screening showed that 4.2% (19/450) of adults aged ≥ 60 years old had detectable nAb titres against the 2024 cattle H5N1. 80% (4/5) of serum specimens with nAb titre of ≥ 40 had detectable Hemagglutination inhibition (HI) titre, and there was a positive correlation between nAb titre and HA binding ($r = 0.3325$, 95% confidence interval 0.2477–0.4123; $P < 0.0001$). For individuals aged ≥ 60 years old, the nAb titre against seasonal H1N1 virus was 4.2-fold higher for those with detectable H5N1 nAb titre than those ≥ 60 years old ones without (geometric mean titre: 89.3 [95% CI 42.9–185.7] vs 21.3 [95% CI 17.3–26.1], $P < 0.0001$), but there was no statistically significant difference between H5N1 and H3N2 nAb titre. There was no difference in demographics, comorbidities and clinical frailty scores between individuals with detectable H5N1 nAb and those without. Our findings suggest that most individuals lack nAb response against 2024 cattle H5N1 and there is an urgency to develop and evaluate H5N1 vaccine or prophylactic monoclonal antibodies. Immune imprinting may be responsible for the cross neutralization between H5N1 and H1N1 among older adults.

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Introduction

The highly pathogenic avian influenza (HPAI) virus A(H5N1) was first documented to cause human infections in Hong Kong in 1997 [1]. Subsequent human infections were primarily confined to Western Pacific Region and Egypt [2,3]. However, the subclade 2.3.4.4b, which has caused widespread infections among aquatic and terrestrial wild mammals, pets, farmed minks and dairy cattle [4], caused an unprecedented human outbreak in North America since March 2024 [5]. As of 17th January 2025, 68 human cases have been reported, including 67 in the United States and 1 in Canada [6,7]. Most cases in this

North American outbreak had exposure to infected dairy cows or poultries [5,8–10]. Two severe cases have been reported. In November, an adolescent was admitted to critical care for respiratory and renal failure [7]. On 18th December 2024, the US Centers for Disease Control and Prevention reported a severe case at Louisiana [11], and the patient died [12].

Several lines of evidence suggest that the clade 2.3.4.4b currently circulating among humans, cattle and other mammals in North America has the potential to become more adapted to humans with pandemic threat. First, unlike earlier H5N1 human infections which were associated with a mortality

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rate approaching 60%, many recent cattle H5N1 human infections have presented with mild respiratory symptoms and conjunctivitis [5,8,9]. Investigations among workers at affected dairy farms suggested that 7% to 14.3% had serological evidence of recent H5N1 infection [13,14]. Given the relatively mild clinical presentation, many cases of human H5N1 infections would be unnoticed, facilitating continual transmission in humans for further adaptation and increasing the risk for a pandemic. Second, an H5N1 strain isolated from a patient in the current 2024 United States outbreak was found to replicate efficiently in primary alveolar epithelial cells and can be transmitted between ferrets via respiratory droplets, direct contact or fomite [15]. Third, some key mutations can have a major impact on transmission and human adaptation. An H5N1 strain with PB2-627 K (A/Texas/37/2024) transmitted more efficiently between ferrets than prior cattle strains without this mutation [15]. A single mutation at haemagglutinin (HA) (G226L [H3 numbering]) can completely switch the receptor binding specificity from avian type to human type receptor [16]. The recent two severe human cases belong to a new genotype D1.1. The H5N1 from the Canadian case has an HA A144 T mutation which can enhance α 2-6 receptor binding [17]. Finally, the H5N1 has been found in a pig in Oregon [18]. Since pigs are well known mixing vessels for different influenza viruses, the ability of H5N1 to infect pig increases the risk of reassortant of H5N1 with seasonal influenza viruses H1N1 or H3N2.

As part of the pandemic preparedness for H5N1, it is essential to estimate the level of population immunity. Neutralizing antibodies (nAbs) are critical in providing protection against influenza viruses [19–21]. In this study, we evaluated the levels of H5N1 nAb in the general population in Hong Kong Special Administrative Region (HKSAR). Our findings indicate that while the majority of individuals lack nAbs against H5N1, a substantial proportion of older adults possess these nAbs, which may be attributable to immune imprinting during childhood.

Methods

Patient specimens

The first part of this study included random anonymized archived serum specimens collected between April and July 2024 from the clinical biochemistry laboratory of Queen Mary Hospital in Hong Kong [21]. For the determination of host factors associated with detectable nAb against 2024 cattle H5N1, we randomly selected serum specimens collected between May and October 2024 from individuals who have been enrolled in our ongoing serological study in older adults, in which we also collected data on

demographics, comorbidities, vaccine history and clinical frailty score [22]. The clinical frailty score was determined using the Clinical Frailty Scale version 2.0 [23]. This study was approved by the Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB) (IRB reference numbers UW 22-328 and UW 18-141).

Viruses

All H5N1 influenza viruses used in this study were rescued using the reverse genetics as we described previously with modifications [24]. The eight gene segments of A/dairy cow/Texas/24-008749-003/2024 (2024 cattle H5N1) (GISAID number: EPI_ISL_19014386) or A/Vietnam/1194/04 (2004 human H5N1) (NCBI: txid644788) were cloned into a pHW2000 plasmid system and used as the backbones. The H1N1 strain (EPI_ISL_19591854) used in the live virus neutralization test was isolated from a nasopharyngeal swab specimen collected in April 2024 and belonged to clade 5a.2a subclade C.1.9, while the H3N2 strain (EPI_ISL_19591855) was isolated from a nasopharyngeal swab specimen collected in January 2024 and belong to clade 2a.3a.1 subclade J.2. All viruses were cultured in Madin-Darby canine kidney (MDCK) cell line (ATCC, Cat# CCL-34). All experiments involving live H5N1 virus were performed in our biosafety level 3 facility.

Live virus neutralization test

Serum specimens were heat inactivated at 56°C for 30 min and were serially diluted in twofolds starting from 1:10. Duplicates of each serum dilution were mixed with 100 TCID₅₀ (50 μ L) of 2024 cattle H5N1 (73 plaque forming units [PFUs]), 2004 human H5N1 (57 PFUs), H1N1 (76 PFUs) or H3N2 (50 PFUs) virus isolates for 1 h, and the serum-virus mixture was then added to MDCK cells. After incubation for 1.5 h, the mixture was removed, and fresh minimum essential medium (MEM) (Gibco, Catalog no. 11095080) or MEM containing 2 μ g/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, Catalog no. T1426) was added to each well. After incubation for 3 days, cytopathic effect (CPE) was examined. The nAb titre was defined as the highest dilution with 50% inhibition of CPE. For the purpose of statistical analysis, a value of 5 was assigned if CPE was observed at a dilution of 1:10. A value of 1280 was assigned if CPE was not observed at a dilution of 1:1280.

Generation of 2024 cattle H5N1 trimer

The expression plasmid vector for 2024 cattle H5N1 was sourced from the Addgene plasmid repository

(pCD5-H3TX12-CO-GCN4-TEV-sfGFP-TS #158739). The expression plasmid was constructed by inserting the vector GCN4-TEV-sfGFP-TwinStrep (C terminal on insert). For protein expression, pCD5-H5N1-GCN4-TEV-sfGFP-TwinStrep plasmid was transfected into HEK293F cells. Culture supernatant was harvested 4 days post-transfection. HA was purified using Strep-Tactin resin columns and nickel affinity columns. TEV protease was used to cleave off the sfGFP fluorescent protein overnight at 4°C. To remove TEV protease and sfGFP fluorescent protein, further purification was performed using Superdex™ 200 Increase 10/300 size-exclusion chromatography (SEC) column. The HA expression was verified with sodium dodecyl sulphate polyacrylamide gel electrophoresis (Supplementary Figure S1).

Enzyme immunoassay (EIA) for 2024 cattle H5 haemagglutinin

The EIA for the 2024 cattle H5 HA protein was performed as we described previously with modifications [25]. Briefly, 96-well half area polystyrene high binding microplates (Corning, Catalog no. 3690) were coated with 50 µL of 2024 cattle H5N1 HA protein (50 ng/well) in 0.05 M NaHCO₃ (pH 9.6) overnight at 4°C. After blocking with blocking reagent overnight at 4°C, 50 µL of heat-inactivated human serum specimens at 1:100 dilution or a mouse anti-H5N1 monoclonal antibody were added to the wells and incubated at 37°C for 1 h. Human or mouse immunoglobulin G (IgG) were detected using horseradish-peroxidase (HRP)-conjugated goat anti-human IgG (Thermo Fisher Scientific, Catalog no. A18847) or goat anti-mouse IgG antibodies (Thermo Fisher Scientific, Catalog no. 31430), respectively. The reaction was developed by adding diluted 3,3',5,5'-tetramethylbenzidine single solution (TMB) (Invitrogen, Catalog no. 002023) and stopped with 0.3 N H₂SO₄. The optical density (OD) was read at 450 and 620 nm.

Hemagglutination inhibition (HI) assay

HI assay was performed as we described previously [26]. Briefly, non-specific inhibitors in sera were removed by incubation with receptor destroying enzyme (RDE) and preadsorption with turkey erythrocyte. Serial 2-fold dilutions of serum from 1:10 were titrated against 4 HA units of A/dairy cow/Texas/24-008749-003/2024 using 0.5% turkey erythrocyte.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 10.4.0 or SPSS version 26. Mann Whitney U test and Fisher's exact test were used for

continuous and categorical variables, respectively. One-way ANOVA test with Tukey's post-hoc test was performed for continuous variables if more than two groups. The correlation between log-transformed nAb titre and HA EIA OD values was computed using Pearson correlation. Log-transformed nAb titre was used statistical analysis. A *P* value of <0.05 was considered statistically significant.

Results

We first screened for nAb against H5N1 viruses among 180 archived anonymized serum specimens collected from June to July 2024, including 20 specimens for each 10-year age cohort from 0–9 to ≥80-year-old cohorts (Supplementary Figure S2). The serum nAb titres against the 2024 cattle H5N1 were determined using live virus neutralization test. Overall, 2.2% (4/180) of serum specimens had detectable nAb against 2024 cattle H5N1 (Table 1). Subgroup analysis showed that the proportion of individuals with detectable nAb against 2024 cattle H5N1 was highest for the ≥80-year-old age cohort (10% [2/20]), followed by 70–79-year-old cohort (5% [1/20]), and the 60–69 year-old cohorts (5% [1/20]). None of the individuals in age cohorts of 50–59 year-old or younger had detectable nAb against the cattle H5N1. Only 1 of 180 (0.6%) serum specimens had detectable nAb against the clade 1 2004 human H5N1 strain.

Table 1. Proportion of individuals with detectable neutralizing antibody against 2024 cattle H5N1 or 2004 human H5N1.

Anonymized serum specimens collected in July 2024	No. of individuals with neutralizing antibody titre of ≥10 (% [95% confidence interval])	
	2024 Cattle H5N1	2004 human H5N1
All age groups (n = 180)	4 (2.2 [0.9–5.6])	1 (0.6 [0.03–3])
0–9 year (n = 20)	0 (0 [0–16])	0 (0 [0–16])
10–19 year (n = 20)	0 (0 [0–16])	0 (0 [0–16])
20–29 year (n = 20)	0 (0 [0–16])	0 (0 [0–16])
30–39 year (n = 20)	0 (0 [0–16])	0 (0 [0–16])
40–49 year (n = 20)	0 (0 [0–16])	0 (0 [0–16])
50–59 year (n = 20)	0 (0 [0–16])	0 (0 [0–16])
60–69 year (n = 20)	1 (5 [0.3–24])	0 (0 [0–16])
70–79 year (n = 20)	1 (5 [0.3–24])	0 (0 [0–16])
80 years or above (n = 20)	2 (10 [1.8–30])	1 (5 [0.3–24])

Table 2. Proportion of anonymized older adults (≥60 years old) with detectable neutralizing antibody against 2024 cattle H5N1 or 2004 human H5N1.

Neutralizing antibody titre	No. of individuals (% [95% confidence interval]) (n = 450)	
	2024 Cattle H5N1	2004 human H5N1
≥10	19 (4.2 [2.7–6.5])	5 (1.1 [0.5–2.6])
≥20	10 (2.2 [1.2–4.0])	1 (0.2 [0.01–1.2])
≥40	5 (1.1 [0.5–2.6])	0 (0.0 [0.0–0.85])
≥80	1 (0.2 [0.01–1.2])	0 (0.0 [0.0–0.85])

The 450 individuals included the 60 individuals aged ≥60 years old in Table 1.

In order to have a more accurate estimate of the proportion of individuals with H5N1 nAb, we tested additional 390 serum specimens collected from individuals ≥ 60 years old between April and July 2024. Out of all 450 anonymized serum specimens collected from ≥ 60 years old (including the 60 serum specimens from ≥ 60 years old in Table 1), 4.2% (19/450) had detectable 2024 cattle H5N1 nAb, with 1.1% (5/450) having an nAb titre of ≥ 40 (Table 2). 16 serum specimens had detectable nAb against 2024 cattle H5N1 but not 2004 human H5N1, 2 had detectable 2004 human H5N1 but not 2024 cattle H5N1, and 3 sera had detectable nAb against 2024 cattle H5N1 and 2004 human H5N1.

Next, we determined whether H5N1 nAb targets the HA. The proportion of individuals with detectable HI titre was significantly higher for sera with 2024 cattle nAb ≥ 10 than those without detectable nAb titre (47.4% [9/19] vs 6.7% [29/431], $P < 0.0001$) (Figure 1). Notably, 4 of 5 (80%) serum specimens with nAb titre of ≥ 40 had detectable HI titre. The 2024 cattle H5N1 nAb titre also positively correlated with the HA binding in the EIA ($r = 0.3325$, 95% confidence interval 0.2477–0.4123; $P < 0.0001$) (Figure 1).

Since both H5 and H1 belong to HA phylogenetic group 1 [27], we hypothesize that the observed H5N1 serum neutralizing activity was related to cross reactivity from H1N1 nAb. To address this hypothesis, we compared the nAb against a 2024 H1N1 strain and a 2024 H3N2 strain of 19 serum specimens with

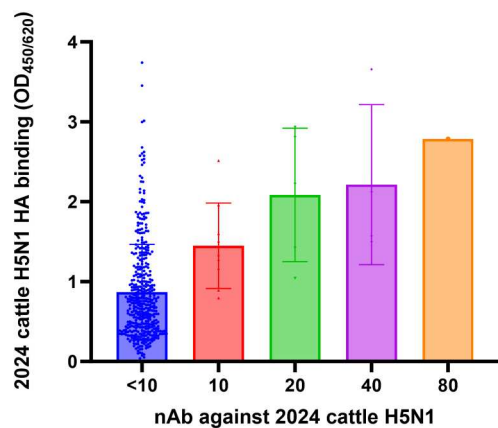


Figure 1. Correlation between 2024 cattle H5N1 nAb titer and binding to 2024 cattle H5 HA, and between 2024 cattle H5N1 nAb and HI titer. HA binding was determined using EIA with 2024 cattle H5 HA trimer. Each bar represents the mean and standard deviation, and each dot represents the optical density value in the HA EIA. The proportion of individuals with 2024 cattle H5N1 HI titer ≥ 10 for each 2024 cattle H5N1 nAb titer is shown in the panel. HA, haemagglutinin; HI, hemagglutination inhibition; nAb, neutralizing antibody; OD, optical density.

detectable cattle H5N1 nAb titre (all ≥ 60 years old), and 180 randomly selected serum specimens without detectable cattle H5N1 nAb titres (90 sera from ≥ 60 years old and 90 from < 60 years old). The H1N1 nAb titre was 4.2-fold higher for ≥ 60 years old individuals with detectable H5N1 nAb titre (geometric mean titre [GMT]: 89.3 [95% CI 42.9–185.7]) than those ≥ 60 years old without detectable H5N1 nAb titre (GMT: 21.3 [95% CI 17.3–26.1]; $P < 0.0001$), and 3.3-fold higher than < 60 years old individuals without detectable H5N1 nAb titre (GMT: 26.8 [95% CI 22.2–32.4], $P < 0.0001$) (Figure 2). However, there was no statistically significant difference in the H3N2 nAb titre between individuals ≥ 60 years old with detectable cattle H5N1 nAb titre than those without. Among the 180 randomly selected serum specimens without detectable cattle H5N1 nAb titres, there was no statistically significant difference in the H1N1 GMT between ≥ 60 year-old and < 60 year-old (GMT: 21.3 [95% CI 17.3–26.1] vs 26.8 [95% CI, 22.2–32.4]; $P = 0.2767$), but H3N2 GMT was 1.4-fold lower for ≥ 60 year-old than < 60 year-old (GMT: 21.3 [95% CI, 17.6–25.7] vs 29.2 [95% CI, 25.4–33.5]; $P = 0.018$).

To determine if there were any host factors that were associated with detectable 2024 cattle H5N1 nAb, we retrieved 100 serum specimens collected from older adults aged ≥ 70 years old between May and October 2024 who were enrolled in ongoing serosurveillance study [22]. We specifically included influenza vaccination history because a previous study showed that seasonal influenza vaccination can influence H5N1 nAb titres [28]. There were no significant differences between those with detectable cattle H5N1 nAb and those without in terms of age, sex, comorbidities, influenza vaccination history and clinical frailty score (Table 3).

Discussion

Serosurveillance has been an indispensable tool to understand population immunity during the A(H1N1)pdm09 and the COVID-19 pandemic [29,30]. In this study, we utilized serosurveillance to evaluate the risk posed by the 2024 clade 2.3.4.4b cattle H5N1 to the general public. Our initial screening of 180 anonymized serum specimens obtained from various age groups revealed that only 2.2% had detectable nAb titres against the 2024 cattle H5N1. These findings underscore the vulnerability of our population to H5N1. Consequently, if the H5N1 were to become more transmissible among humans, there is a possibility of rapid surge in patients that could potentially overwhelm the healthcare system, similar to the situation for A(H1N1)pdm09 and COVID-19 pandemics.

H1N1, but not H3N2, nAb titre was statistically significantly higher among those with detectable H5N1 nAb. Our results suggest that there are cross reactive

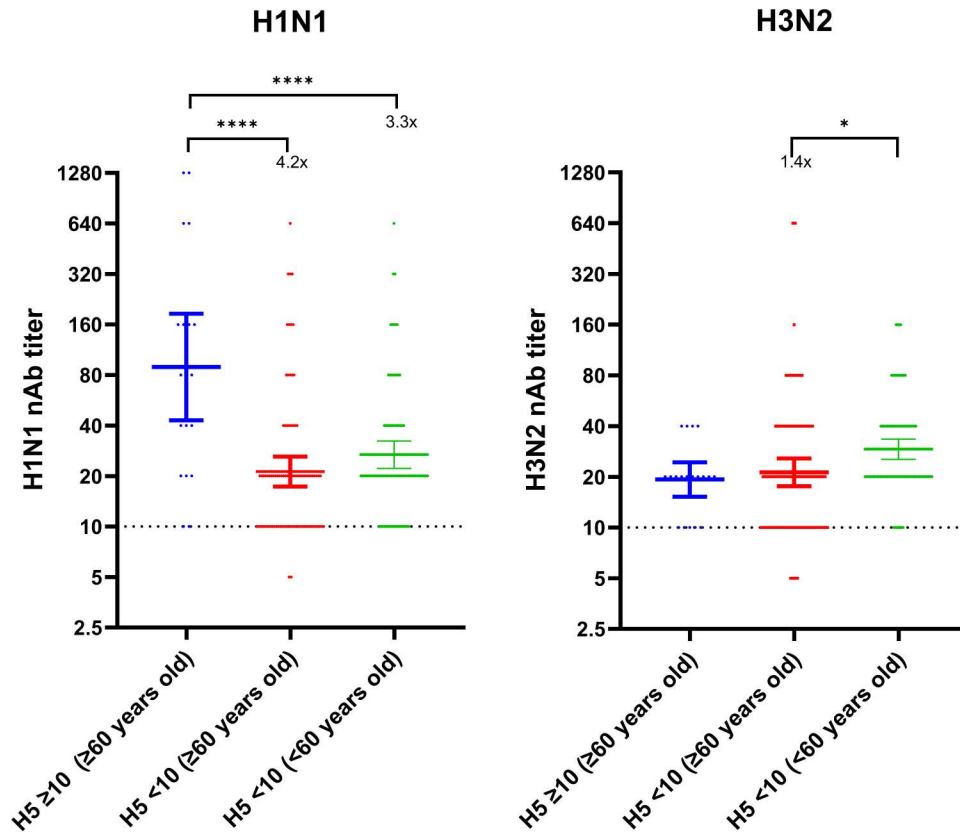


Figure 2. Comparison of nAb titre against seasonal influenza viruses between patients with detectable nAb against 2024 cattle H5N1 and those without detectable nAb against 2024 cattle H5N1. (A) H1N1; (B) H3N2. Dotted line indicates the lower detection limit. Horizontal bars represent the geometric mean nAb titre with 95% confidence interval. Statistical comparison was performed using one-way ANOVA test with Tukey's multiple comparison. *, $P < 0.05$; ****, $P < 0.0001$.

Table 3. Host factors associated with detectable 2024 cattle H5N1 nAb.

	2024 cattle H5N1 nAb titre		<i>P</i> value ^a
	<10 (n = 90)	≥10 (n = 10)	
Demographics			
Median age (range)	73 (70–75)	71 (70–75)	0.106
Female sex	56 (62.2)	9 (90)	0.159
Comorbidities			
Hypertension	64 (71)	7 (70)	1.000
Heart disease	50 (56)	5 (50)	0.750
Lung disease	9 (10)	1 (10)	1.000
Liver disease	18 (20)	2 (10)	1.000
Kidney disease	7 (7.8)	1 (10)	0.583
Autoimmune disease	1 (1)	1 (10)	0.191
Haematological disorders	13 (14)	2 (20)	0.643
Malignancy	13 (14)	1 (10)	1.000
Diabetes mellitus	30 (33)	3 (30)	1.000
Thyroid disease	12 (13)	0 (0)	0.604
Influenza vaccine			
2020–2021 season	51 (57)	6 (60)	1.000
2021–2022 season	46 (51)	4 (40)	0.741
2022–2023 season	55 (61)	6 (60)	1.000
2023–2024 season	71 (79)	7 (70)	0.687
2024–2025 season	15 (17)	1 (10)	1.000
Have not received influenza vaccine from 2020 to 2024	14 (15.6)	3 (30)	0.367
Median clinical frailty score (range)	3 (2–5)	3 (2–3)	0.656

Data expressed as no. (%) unless otherwise stated.

SD: standard deviation.

^aFisher's exact test for categorical variables; Mann Whitney U test for continuous variables.

nAb in these serum specimens that neutralize both H1N1 and H5N1. Both HA and neuraminidase (NA) can be the target of broadly reactive nAb. For HA, previous studies demonstrated that the stalk region and the vestigial esterase domain of the HA protein are the targets of broadly reactive Ab that neutralizes both H5N1 and H1N1 [31,32]. For NA, prophylactic treatment with anti-NA monoclonal antibodies, which targets the lateral face of NA, could protect mice against lethal H1N1 or H5N1 infection [33]. NA is also a target of broadly neutralizing monoclonal antibodies which can protect mice from both H1N1 and clade 2.3.4.4b H5N1 virus [34]. Future studies should assess the role of NA nAb in the neutralization of H5N1 viruses.

Age-stratified analysis indicated that all serum specimens with detectable cattle H5N1 nAb were obtained from individuals aged 60 years or above, while none of the 120 individuals aged between 0 and 59 years old had detectable cattle H5N1 nAb. Our results suggest that immune imprinting due to childhood exposure to H1N1 or H2N2 may elicit nAb that cross neutralize H5N1. Our results are consistent with the observations in previous studies. Gostic et al. demonstrated that childhood imprinting with H1N1

was associated with 75% protection against severe H5N1 infection [35]. A recent study by Garretson et al. demonstrated that individuals born before 1968 had higher levels of H5N1 HA binding Ab [36]. Supporting this, a study in ferrets showed that immune imprinting with H1N1 increases the survival rates for H5N1 infection [37]. Similarly, in mice infected with H5N1, those primed with H1 HA had less weight loss when compared to those primed with H3 HA [38].

Another possible reason for having H5N1 nAb is repeated vaccination against influenza virus. Using pseudovirus neutralization test, Wang et al. showed that 36% of adults aged 48–64 years old who have received multiple seasonal influenza vaccines from 2004 to 2009 had nAb of ≥ 160 against the H5N1 Vietnam/1203/04, though none had nAb of ≥ 160 against several other H5N1 strains isolated from 2002 to 2006 [28]. However, in our study, we did not observe any statistically significant difference among individuals who have received influenza vaccine and those who did not. Therefore, it is unlikely that repeated seasonal influenza vaccination can reliably elicit H5 nAb.

There are several limitations. First, this study was conducted in HKSAR where H5N1 and other subtypes of avian influenza viruses exposure may be more frequent than places without H5N1 circulation, especially before the implementation of stringent live poultry market measures [2]. Hence, there is a possibility that some individuals may indeed have prior exposure to H5N1 or H5Nx. However, it should be noted that low levels of H5N1 nAb can be found in individuals recruited for studies in the United States before the 2024 H5N1 outbreak [36]. Second, caution is warranted when comparing our results from those of others, as different assays were employed. In our current study, we used a conventional live virus neutralization test. However, other studies have used pseudovirus neutralization test or HI assay only [13,14]. Third, we have not explored the role of NA in immune imprinting.

Our serosurveillance study showed that our general population is vulnerable to H5N1 infection. A small proportion of older adults have cross-neutralizing antibody against H5N1, likely due to childhood immune imprinting. Currently, 2 of 65 (3.1%) of cases in the North American clade 2.3.4.4b outbreak require critical care. The clade 2.3.4.4b H5N1 virus can cause fatal infection in ferrets and mice [15,39]. There is an urgent need to develop and evaluate H5N1 vaccine against the clade 2.3.4.4b before this virus becomes pandemic.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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