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Serological evidence of influenza virus infection in captive wild felids, Thailand

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ABSTRACT. Influenza virus is known to affect wild felids. To explore the prevalence of influenza viruses in these animal species, 196 archival sera from 5 felid species including *Panthera tigris* (N=147), *Prionailurus viverrinus* (N=35), *Panthera leo* (N=5), *Pardofelis temminckii* (N=8) and *Neofelis nebulosa* (N=1) collected between 2011 and 2015 in 10 provinces of Thailand were determined for the presence of antibody to avian and human influenza viruses. Blocking enzyme-linked immunosorbent (ELISA) assay and hemagglutination inhibition (HI) assay were employed as the screening tests, which the serum samples with HI antibody titers ≥20 were further confirmed by cytopathic effect/hemagglutination based-microneutralization (CPE/HA-based microNT) test. Based on HI and microNT assays, the seropositive rates of low pathogenic avian influenza (LPAI) H5 virus, highly pathogenic avian influenza (HPAI) H5 virus and human H1 virus were 1.53% (3/196), 2.04% (4/196) and 6.63% (13/196), respectively. In addition, we also found antibody against both LPAI H5 virus and HPAI H5 virus in 2 out of 196 tested sera (1.02%). Evidences of influenza virus infection were found in captive *P. tigris* in Kanchanaburi, Nakhon Sawan and Ratchaburi provinces of Thailand. The findings of our study highlights the need of a continuous active surveillance program of influenza viruses in wild felid species.

KEY WORDS: captive wild felid, influenza virus, serosurveillance

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Influenza viruses belong to the family *Orthomyxoviridae.* The viruses are divided into 4 different types including influenza A, B, C and D. Only influenza A viruses (IAV) are able to infect a broad range of host species including humans and animals. The continuous circulation in animal hosts could make the influenza viruses acquire to undergo periodic genetic changes, leading to the possibility of the emergence of the new strains with epidemic and pandemic potential [16]. Several serious widespread infection in the past had been caused by influenza viruses with zoonotic origin such as the swine-origin H1N1 virus and the avian-origin H5N1 virus [18, 21].

The role of animal hosts in the continued evolution of influenza viruses has been more concern, which the targeted species also include wildlife populations. The captive wild felids particularly tigers are known to be infected by influenza viruses. The first report in 2002 identified the infection of the highly pathogenic avian influenza (HPAI) H5N1 virus in a tiger (*Panthera tigris*) in China [27] and the virus still occasionally been found in this animal species in China as previously described [8, 9, 17]. The HPAI H5N1 virus had been detected in captive tigers and leopards in Suphanburi province, central Thailand and Chonburi province, eastern Thailand in 2004 during outbreaks in Thailand [2, 11, 22]. Recently, the evidence of seropositive to HPAI H5 virus and the low pathogenic avian influenza (LPAI) H9 virus was found in captive Siberian tigers in Northeastern China [24].

In addition to the avian influenza viruses, several studies demonstrated the transmission of human influenza viruses such as the 2009 pandemic H1N1 virus to a variety of animal species [1, 6, 7, 12, 14, 20]. Confine to captive wild felids especially tigers, the animals can possibly expose to the virus through the close contact with the infected caretakers or tourists who engage the activities such as playing or feeding the animals. However, little has been known about human influenza virus infection in these animals.

To better understand the potential exposure of influenza virus in captive wild felids, serosurveillance for avian and human influenza viruses was conducted in 5 species of captive wild felids including tigers and lions using archival serum samples collected between 2011 and 2015 in 10 provinces of Thailand. The blocking enzyme-linked immunosorbent (ELISA) assay

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and hemagglutination inhibition (HI) assay were used as the screening tests; while cytopathic effect/hemagglutination based-microneutralization (CPE/HA-based microNT) assay was used as the confirmatory test.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Ethics and Animal Care and Use Committee of the Faculty of Veterinary Science, Mahidol University (Permit Number: MUVS-2018-01-04).

Archival captive wild felid sera

A total of 196 serum samples were collected between 2011 and 2015 from 5 captive felid species including *Panthera tigris*, *Panthera leo*, *Pardofelis temminckii*, *Prionailurus viverrinus* and *Neofelis nebulosa*, which the habitats of the animals were distributed in 10 provinces of Thailand (Fig. 1 and Table 1). All archival sera were kept at −80°C until use. Most of the captive wild felids showed no respiratory diseases or clinical symptoms at the time of blood collection.

The study viruses

In this study, both avian and human influenza viruses were employed for HI and microNT tests. For avian influenza viruses, 5 subtypes of LPAI (H1, H3, H5, H7 and H9) and 1 subtype of HPAI (H5) viruses were chosen based on the following considerations. The HPAI H5 virus and LPAI H9 virus have been detected in tigers earlier [24] while various LPAI subtypes including H1, H3, H4, H7, H8, H10 and H11 have been identified in domestic avian species in Thailand [4, 5, 10, 26]. Furthermore, the potential of LPAI H5 and H7 viruses to evolve into HPAI viruses has raised concerns which the circulation of these subtypes should be monitored in animal reservoirs especially in the avian population. In addition, due to the limitation of the tiger serum amount, some of the relevant subtypes of avian influenza viruses were selected as the tested antigen.

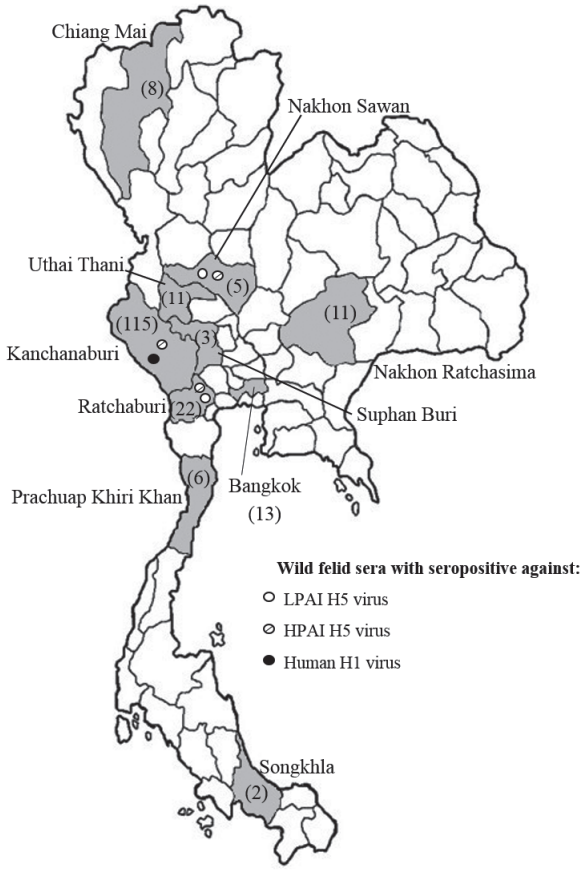

Fig. 1. Geographical distribution of the sera collected from captive wild felids. The total number of the sera obtained from each province is shown in the parentheses. The seropositive sera against influenza subtypes are indicated. LPAI, low pathogenic avian influenza; HPAI, highly pathogenic avian influenza.

Five subtypes of LPAI viruses including A/Aquatic bird/Hong Kong/DI25/2002 (H1N1), A/Duck/Shan Tou/1283/2001 (H3N8), A/Duck/Jiangxi/6151/2003 (H5N3), A/Ostrich/Zimbabwe/222/96 (H7N1) and A/Chicken/Hong Kong/G9/97 (H9N2) were kindly provided by St. Jude Children Research Laboratory, Memphis, TN, U.S.A. through Professor Emeritus Dr. Pilaipan Puthavathana. The HPAI virus used in this study, A/Chicken/Thailand/VSMU-3-BKK/2004 (H5N1) (H5N1 virus clade 1) was isolated by our laboratory. All viruses were propagated in embryonated eggs to prepare the virus stocks for using in the further assays. The manipulation of the above listed viruses was conducted at the biosafety level 3 (BSL-3) facility of Faculty of Veterinary Science, Mahidol University.

Regarding to human influenza viruses, the 2009 pandemic H1N1 virus was chosen for this study because of the evidences of the transmission of virus to a variety of animal species. The pandemic human influenza virus used in this study, A/Thailand/104/2009 (H1N1), was propagated in Madin-Darby canine kidney (MDCK) cells (obtained from the American Type Culture Collection; CCL-34) maintained in the viral growth media containing Eagle's minimum essential medium (EMEM) (Gibco, Grand Island, NY, U.S.A.) supplemented with 2 μg/m*l* of trypsin-tosyl phenylalanyl chloromethyl ketone (trypsin-TPCK) (Sigma-Aldrich, St. Louis, MO, U.S.A.). The propagation of the virus was carried out at the BSL-2 facility of our faculty.

Blocking enzyme-linked immunosorbent (ELISA) assay

All archival sera were tested for influenza A virus (IAV) antibodies using a commercially species-independent test kit, IDEXX Influenza A Ab test kit (IDEXX Laboratories Inc., Westbrook, ME, U.S.A.). The test was used according to the manufacturer's instructions. Briefly, 15 μl of each sample was diluted in 135 μl of dilution buffer. A 100 μl of each diluted sample, undiluted positive and negative controls were dispensed into the nucleoprotein (NP) antigen coated plate. The plate was incubated for 60 min at 18–26°C and washed with washing solution. A 100

Table 1. Demographic characteristics of archival sera derived from captive wild felids

Year	Species	Province	Place	Number
2011	*Panthera tigris*	Kanchanaburi	Wildlife sanctuary area	89
	Prionailurus viverrinus	Prachuap Khiri Khan	Unknown place	4
	Panthera leo	Kanchanaburi	Wildlife sanctuary area	2
2012	*Panthera tigris*	Bangkok	Marine and safari park	2
		Ratchaburi	Wildlife breeding center	6
		Uthai Thani	Unknown place	11
	Prionailurus viverrinus	Ratchaburi	Wildlife breeding center	3
	Pardofelis temminckii	Ratchaburi	Wildlife breeding center	8
	Panthera leo	Suphan Buri	Zoo and aquarium	3
2013	*Panthera tigris*	Kanchanaburi	Wildlife sanctuary area	22
		Kanchanaburi	Safari park	2
		Nakhon Sawan	Unknown place	5
		Ratchaburi	Wildlife breeding center	4
	Prionailurus viverrinus	Prachuap Khiri Khan	Marine national park	2
2014	*Panthera tigris*	Ratchaburi	Wildlife breeding center	1
	Prionailurus viverrinus	Bangkok	Zoo of central Thailand	7
	Neofelis nebulosa	Bangkok	Zoo of central Thailand	1
		Chiang Mai	Zoo of northern Thailand	5
2015	*Prionailurus viverrinus*	Bangkok	Zoo of central Thailand	3
		Chiang Mai	Zoo of northern Thailand	3
		Nakhon Ratchasima	Zoo of northeastern Thailand	11
		Songkhla	Zoo of southern Thailand	2
Total				196

μl of monoclonal anti-influenza A NP conjugate was added into each well, incubated for 30 min at 18–26°C and washed again. A 100 *μl* of TMB substrate solution was dispensed into each well, incubated for 15 min in the dark area and then added 100 *μl* of stop solution. To obtain the S/N ratio, the OD value of the sample was divided by the OD value of the negative control after measuring at 650 nm using an ELISA reader (Biotek Instruments Inc., Winooski, VT, U.S.A.). If the S/N ratio is <0.60, the sample is considered as IAV antibody positive. Conversely, if S/N ratio is ≥0.60, it is interpreted as IAV antibody negative.

Hemagglutination (HA) assay

The test viruses were determined for hemagglutination titer prior to performing HI assay. In HA assay, the test viruses were serially 2-fold diluted with phosphate buffer saline (PBS) pH 7.2 in a volume of 50 *μl*/well in duplicate. A 50 *μl* volume of 0.5% goose erythrocyte suspension was then added and incubated for 30 min at 4°C before determining the HA titer. One HA unit of the test virus was defined as the highest virus dilution that yield complete hemagglutinating activity.

Hemagglutination inhibition (HI) assay

All test sera were treated with a receptor-destroying enzyme (RDE) and absorbed by the goose erythrocytes to remove non-specific inhibitors and non-specific agglutinators. A 20 *μl* volume of the test serum was mixed with 60 *μl* of RDE (Denka Seiken Co., Ltd., Tokyo, Japan) and incubated overnight in a water bath at 37°C. The mixture was then heat-inactivated for 30 min at 56°C to inactivate the RDE, followed by absorbing with 20 *μl* of 50% goose erythrocyte suspension for 1 hr at 4°C. The serum samples were centrifuged at 3,000 × g for 10 min and collected the supernatant. The RDE-treated serum was added with NSS to obtain the dilution of 1:10.

Five subtypes of LPAI virus, H5N1 subtype of HPAI virus and human H1N1 virus were titrated as described above, and used as the test antigen at a final concentration of 4 HA units/25 *μl*. HI assay was performed as previously described [13, 15]. A 25 *μl* volume of the treated serum was serially 2-fold diluted in PBS pH 7.2 in duplicate wells of V-bottom shaped microtiter plates at an initial serum dilution of 1:20, and then added with 4 HA units of the test virus. The reaction plates were incubated at room temperature for 30 min, and added with 50 *μl* of 0.5% goose erythrocyte suspension, and further incubated for 30 min at 4°C before determining the HI antibody titer. The reciprocal of the highest serum dilution that completely inhibit virus-mediated agglutination of goose erythrocytes was assigned as the HI antibody titer. For calculation of geometric mean titer (GMT), the HI titer <20 was assigned as 10. An HI titer ≥20 was considered as seropositive and indicated previous infection.

Microneutralization (microNT) assay

The positive samples obtained from the HI assay were further determined for neutralizing (NT) antibody using microNT assay. The protocol for microNT assay was followed by that previously described [13, 19]. Briefly, 60 *μl* of the treated serum was serially 2-fold diluted in EMEM (Gibco) in 96-well microculture plates in duplicate at an initial dilution of 1:20 and then added the tested

virus at the final concentration of 100 TCID50/well. The reaction plates were incubated for 2 hr at 37°C and then transferred the serum-virus mixture onto MDCK monolayer maintained in EMEM supplemented with trypsin-TPCK for LPAI virus and without trypsin-TPCK for HPAI virus. The MDCK monolayer were further incubated at 37°C for 2–3 days before observing the appearance of CPE; while the culture supernatants were determined for non-neutralized viruses by HA assay. The reciprocal of the highest serum dilution that inhibited the degree of CPE by 50% and showed ≤2+ degree of hemagglutination was defined as the NT antibody titer.

RESULTS

Blocking ELISA for IAV antibody detection

A total of 196 archival captive wild felid sera were screened for the presence of IAV antibody using IDEXX Influenza A Ab test kit, species-independent test kit, which target conserved NP. There were 15 (7.65%) captive wild felids that contained IAV antibody positive. The prevalence of IAV antibody detected from the felid serum corresponding to the year of serum collection were 3.16% (3/95) in 2011, 12.12% (4/33) in 2012 and 22.86% (8/35) in 2013. No IAV antibody was found from the serum collected in 2014 and 2015. The positive serum samples were obtained from 2 species of captive wild felids: *P. tigris* living in Kanchanaburi, Nakhon Sawan and Ratchaburi; *P. temminckii* living in Ratchaburi province of Thailand (Tables 2 and 4).

HI assay for antibody to influenza viruses

A total of 196 archival captive wild felid sera were screened for the presence of antibody to 7 subtypes of influenza virus including H1, H3, H5, H7 and H9 subtypes of LPAI virus, H5 subtype of HPAI virus and human H1 virus by using HI assays. The results demonstrated that 18 (9.18%) of 196 serum samples were IAV seropositive with 1.53% (3/196) in LPAI H5 virus, 2.04% (4/196) in HPAI H5 virus and 6.63% (13/196) in human H1 virus, and the seropositivity rate of LPAI H5 virus + HPAI H5 virus was 1.02% (2/196). The overall GMT of LPAI H5 virus, HPAI H5 virus and human H1 virus were 10.14, 10.21 and 11.36, respectively. In addition, the result was negative for antibody to the other subtypes of LPAI including H1, H3, H7 or H9 virus. The prevalence of IAV antibody detected from the felid sera corresponding to the year of serum collection was 6.32% (6/95) in 2011, 6.06% (2/33) in 2012 and 34.29% (12/35) in 2013. The IAV antibody was not detected from the sera collected in the year 2014 and 2015. The positive serum samples were obtained from *P. tigris* living in Kanchanaburi, Nakhon Sawan and Ratchaburi provinces of Thailand (Fig. 1, Tables 3 and 4).

HI and microNT assays for antibody to influenza viruses

The 18 serum samples with HI titer ≥20 were further confirmed for neutralizing antibody by microNT assay. The result demonstrated that all of the tested serum samples had NT antibody titers ≥40. The NT antibody titers in the range of 80–320 (GMT=201.59), 80–640 (GMT=190.27) and 40–640 (GMT=198.04) were found in LPAI H5 virus, HPAI H5 virus and human H1 virus, respectively. Among those 18 serum samples, there were 2 serum samples from the collection in 2013 obtained from captive *P. tigris* at Nakhon Sawan province showing the HI and NT antibody titers to both LPAI H5 virus and HPAI H5 virus. In this case, the HI/NT antibody titer to LPAI H5 virus of the 2 samples were 20/320 and 40/320 while the HI/NT antibody titer to HPAI H5 virus of the 2 samples were in the same titer values at 20/160. The summary of the HI and NT antibody titers of each positive samples was shown in Table 4 and the correlation between those antibody titers to subtypes of influenza viruses were shown in Fig. 2.

DISCUSSION

Most of influenza virus surveillances were conducted in poultry and domestic animals while the surveillance in wildlife species still be limited [25]. To expand the surveillance information of influenza virus infection in wildlife species, we chose the captive

Table 2. Screening for antibody to influenza virus in captive wild felids by blocking enzyme-linked immunosorbent (ELISA) assay

Year	N	Number of sera with IAV antibody positive (%)	Host species of positive samples (N) / locations
2011	95	3 (3.16)	*Panthera tigris* (3) / Kanchanaburi
2012	33	4 (12.12)	*P. tigris* (3) / Ratchaburi *P. temminckii* (1) / Ratchaburi
2013	35	8 (22.86)	*P. tigris* (7) / Kanchanaburi *P. tigris* (1) / Nakhon Sawan
2014	14	0	-
2015	19	0	-
Total	196	15 (7.65)	

IAV, influenza A virus.

wild felids in Thailand for the serological investigation in the present study. Our study demonstrated that 18 of 196 captive felid serum samples (9.18%) were IAV seropositive based on HI and microNT assays. We found antibody titer against subtype of influenza viruses including LPAI H5 virus (3/196, 1.53%), HPAI H5 virus (4/196, 2.04%), human H1 virus (13/196, 6.63%) and both LPAI H5 virus and HPAI H5 virus (2/196, 1.02%). The seropositive sera were found in captive *P. tigris* which located in Kanchanaburi, Nakhon Sawan and Ratchaburi provinces of Thailand.

In this study, we performed the influenza virus antibody detection in felid serum samples by using species-independent blocking ELISA test kit and HI assay for the screening tests and microNT assay for the confirmatory test. The seroprevalence to influenza virus was 7.65% (15/196) obtained by the ELISA test kit and 9.18% (18/196) obtained by HI assay. Of those 15 ELISA positive samples, 13 sera were also found to be positive for influenza virus antibody by HI assay, which the antibody titer were ≥20

Table 3. Screening for antibody to influenza viruses in captive wild felids by hemagglutination inhibition (HI) assay

Year	N	Number of positive sera with HI titers of ≥20 by each subtype of influenza virus							Host species of positive samples (N) / locations / virus subtype infection
		LPAI					HPAI	Human	
		H1	H3	H5	H7	H9	H5	H1	
2011	95	0	0	0	0	0	0	6	*P. tigris* (6) / Kanchanaburi / Human H1
2012	33	0	0	1	0	0	1	0	*P. tigris* (1) / Ratchaburi / LPAI H5 *P. tigris* (1) / Ratchaburi / HPAI H5
2013	35	0	0	2	0	0	3	7	*P. tigris* (2) / Nakhon Sawan / LPAI H5[a)] *P. tigris* (1) / Kanchanaburi / HPAI H5 *P. tigris* (2) / Nakhon Sawan / HPAI H5[a)] *P. tigris* (7) / Kanchanaburi / Human H1
2014	14	0	0	0	0	0	0	0	-
2015	19	0	0	0	0	0	0	0	-
Total	196	0	0	3	0	0	4	13	

a) Two samples from *Panthera tigris* at Nakhon Sawan were seropositive for both low pathogenic avian influenza (LPAI) H5 virus and highly pathogenic avian influenza (HPAI) H5 virus.

Table 4. Serological test results by using enzyme-linked immunosorbent (ELISA), hemagglutination inhibition (HI) and microneutralization (microNT) assays in each positive sample

No.	Blocking ELISA	HI antibody titers[a)]			NT antibody titers[a)]		
	Influenza virus (Nucleoprotein)	LPAI virus (H1, H3, H5, H7 and H9)	HPAI virus H5	Human virus H1	LPAI virus (H1, H3, H5, H7 and H9)	HPAI virus H5	Human virus H1
1	-	-	-	20	-	-	40
2	+	-	-	20	-	-	80
3	-	-	-	20	-	-	80
4	-	-	-	20	-	-	160
5	+	-	-	40	-	-	160
6	-	-	-	80	-	-	160
7	+	-	-	80	-	-	320
8	+	-	-	160	-	-	160
9	+	-	-	160	-	-	320
10	+	-	-	160	-	-	320
11	+	-	-	160	-	-	320
12	+	-	-	160	-	-	640
13	+	-	-	160	-	-	640
14	+	20 (H5)	-	-	80 (H5)	-	-
15	+	-	20	-	-	80	-
16	+	-	80	-	-	640	-
17[b)]	+	20 (H5)	-	-	320 (H5)	-	-
		-	20	-	-	160	-
18[b)]	-	40 (H5)	-	-	320 (H5)	-	-
		-	20	-	-	160	-
19	+	-	-	-	ND	ND	ND
20	+	-	-	-	ND	ND	ND

a) HI and NT antibody titer <20 was considered as seronegative; +: positive, -: negative, ND: not determined. b) Individual tiger serum sample was seropositive to both low pathogenic avian influenza (LPAI) H5 virus and highly pathogenic avian influenza (HPAI) H5 virus.

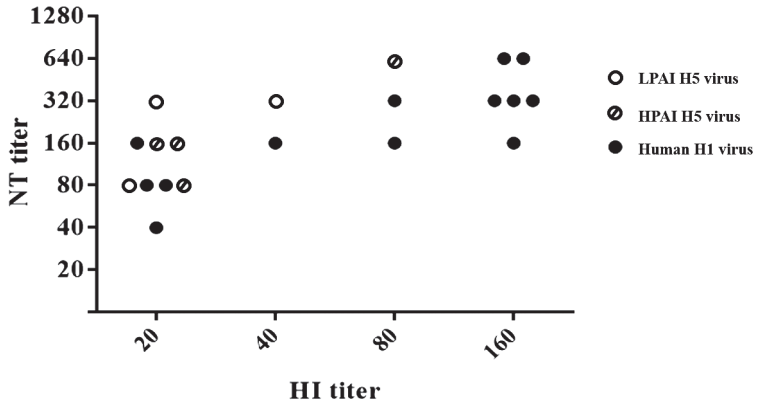

Fig. 2. The correlation between hemagglutination inhibition (HI) and neutralization (NT) antibody titers to influenza viruses including low pathogenic avian influenza (LPAI) H5 virus, highly pathogenic avian influenza (HPAI) H5 virus or human H1 virus of the 18 serum samples. One dot represents individual serum sample except 2 of those 18 samples that are found HI and NT antibody titers to both LPAI H5 virus and HPAI H5 virus.

whereas the other 2 sera were considered to be negative by HI assay due to the antibody titer were <20. Interestingly, we found the additional 5 sera had HI antibody titers ≥20 but all of those sera were negative by ELISA test. When we confirmed the HI positive samples by microNT assay, the NT antibody titers could be observed in the range of 40–640, which the 5 sera with negative screening by ELISA showed NT antibody titers in the range of 40–160 (Table 4). In this case, the 5 sera which were HI positive but ELISA negative should be considered as truly positive. On the other hand, the sera showing HI negative but ELISA positive should be careful for the interpretation. The positive ELISA could probably truly positive if the antibody presented in the sera is specific to other influenza viruses that are not included in the HI panel. However, the use of ELISA test kit as a primary screening tool for serological surveillance of influenza virus should be evaluated in particular when the tested sera were derived from multi-animal species.

Our long-term serological evidence demonstrated the past infection of avian and human influenza viruses in captive *P. tigris* in Thailand. For avian influenza virus exposure, the adult animals were presumably undergone the period of the presence of avian influenza viruses in poultry population (Personal communication). In Thailand, the presence of HPAI H5N1 were estimably the year 2004–2008 [3, 23] while the presence of LPAI subtypes still be reported occasionally from domestic avian species [4, 5, 10, 26]. The most likely possibility of tigers exposure to the viruses is the tigers had been fed raw chicken carcasses that were possibly contaminated with avian influenza viruses. On the other hand, the source of human H1 virus infection in captive *P. tigris* was likely came from the close contact with infected caretakers or tourists who engaged in activities such as playing or feeding with the tigers. Upon available data on the tiger age, 10 out of 13 seropositive sera belonged to the young tigers with the ages ranged from 0.4 to 1.3 years. Moreover, the result indicated that human H1 virus infection was common in tiger cubs originally from Kanchanaburi province, western Thailand in 2011 and 2013. Our previous study demonstrated that the source of human H1 viral infection in Thai elephants likely came from mahouts or tourists who engaged in activities involving with elephants [19].

Our serological findings in captive wild felids implied the susceptibility of influenza virus infection in captive *P. tigris* and highlighted the fact that LPAI and HPAI subtypes of avian influenza viruses as well as human influenza virus can infect tigers. Although the source of influenza virus infection was underexplored, the information on the past exposure of tigers to varied subtypes of influenza viruses could raise public concerns, in particular the human and animal health sectors. The important implications provided by our study could enhance a continuous active surveillance program and epidemiological studies of influenza viruses in wild animals.

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