

Surveillance for influenza virus in nonhuman primates (NHPs) in Thailand, 2009-2018

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Abstract

The present study conducted a longitudinal serological survey and active surveillance for influenza virus in free-ranging nonhuman primates (NHPs) in Thailand. One hundred and nine serum samples from NHPs were collected between 2009 and 2017, in 4 provinces of Thailand; while 282 oropharyngeal swabs were collected between April to August, 2018, in 8 provinces of Thailand. Our results demonstrated that no antibody to influenza virus was found in NHP sera by using blocking enzyme-linked immunosorbent (ELISA) assay and hemagglutination inhibition (HI) assay. Moreover, no influenza genome could be detected in all new NHP swab samples by using real-time RT-PCR assay. However, monitoring and surveying of influenza virus in these species should be maintained.

Keywords: influenza virus, nonhuman primates, surveillance

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Introduction

Influenza A virus causes substantial illness and death among mammals and birds. The virus is known to infect a wide range of host species. The infection in various hosts might contribute the changes of periodic viral genetic, leading to influenza epidemic and pandemic potential (Medina, 2011). Numerous animal species, including domestic and wild animals, can serve as reservoirs, making the control and prevention of virus infection more difficult (Bailey *et al.*, 2018). Although influenza A virus infection has been studied in variety of animal species, the virus exposure in wildlife and exotic animals is still under exploration. Confined to nonhuman primates (NHPs), these animal species have physiological and immunological similarities to humans, thus, allowing them to be used as animal models to study the human response of influenza virus infection (Bouvier, 2010; Davis *et al.*, 2015). However, little is known about natural infection by the influenza virus in the NHP population. Evidence of seropositivity to seasonal human influenza subtypes H1 and H3 virus has been reported in macaques in Bangladesh, Singapore, Java and Sulawesi in Indonesia (Karlsson *et al.*, 2012); and also, in captive chimpanzees in the Netherlands (Buitendijk *et al.*, 2014). Moreover, the presence of antibody against avian influenza subtype H9 virus has also been detected in macaques in Bangladesh (Karlsson *et al.*, 2012). On the other hand, the influenza virus genome has been found in a buccal swab from an adult macaque in Cambodia (Karlsson *et al.*, 2012). In Thailand, the information on influenza virus infection in NHPs has never been explored. It is important to investigate the role of these animals in influenza epidemiology. Especially, at present, the NHP population is growing rapidly in many parts of Thailand where some NHP colonies have their habitats overlap with or be close to the communities. Here, we performed longitudinal serosurveillance using blocking enzyme-linked immunosorbent (ELISA) assay and hemagglutination inhibition (HI) assay and active surveillance using the real-time reverse transcription-PCR technique.

Materials and Methods

Ethics statement: The protocol for this project 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 NHP sera: As part of longitudinal serosurveillance, a total of 109 archival NHP sera including *Macaca fascicularis* (n=62), *Hylobates lar* (n=20), *Pongo pygmaeus* (n=17), *Nycticebus coucang* (n=9), and *Macaca nemestrina* (n=1) were collected between 2009 and 2017 in 4 provinces including Chachoengsao, Kanchanaburi, Ratchaburi and Phangnga provinces of Thailand (Fig 1). These archival sera were obtained from the disease surveillance program of the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University. Some archival sera were obtained from cooperation

with the Department of National Parks, Wildlife and Plant Conservation. The clinical symptoms involving influenza virus infection at the time of blood collection in these NHPs was not observed. All archival sera were kept at -80°C until use.

Oropharyngeal swabs: As part of the active surveillance, oropharyngeal swabs were collected from 282 free-ranging NHPs including *M. fascicularis* (n=247) and *M. mulatta* (n=35) from April to August, 2018, in 8 provinces: Amnat Charoen, Chiang Rai, Lopburi, Mukdahan, Phatthalung, Ratchaburi, Samut Sakhon and Songkhla (Fig 1). The collection of swab samples was undertaken under the neutering program for NHPs by the Department of National Parks, Wildlife and Plant Conservation. The swabs were immediately placed into lysis buffer and transported to the laboratory.

Blocking enzyme-linked immunosorbent (ELISA) assay: All archival sera (n=109) were initially screened for anti-influenza A nucleoprotein antibodies using a commercially species-independent test kit, IDEXX Influenza A Ab test kit (IDEXX Laboratories Inc, Westbrook, ME). The test was used according to the manufacturer's instructions. The threshold of sample result to negative control (S/N) ratio, <0.6 as IAV antibody positive was considered.

Hemagglutination inhibition (HI) assay: All archival sera (n=109) were subjected to HI assay with subtype of pandemic human influenza virus, A/Thailand/104/2009(H1N1) which was kindly provided by Professor Emeritus Dr. Pilaipan Puthavathana. Moreover, 3 subtypes of low pathogenic avian influenza (LPAI) virus including A/Aquatic bird/Hong Kong/DI25/2002(H1N1), A/Duck/Shan Tou/1283/2001(H3N8), and A/Ostrich/Zimbabwe/222/96(H7N1) which were kindly provided by St. Jude Children Research Laboratory, Tennessee, USA. through Professor Emeritus Dr. Pilaipan Puthavathana; and subtype of HPAI virus which was isolated by our laboratory, A/Chicken/Thailand/VSMU-3-BKK/2004(H5N1) were included as tested antigens. HI assay was performed as previously described (Louisirirotchanakul *et al.*, 2007; Lerdamran *et al.*, 2011). The HI antibody titer was defined as the highest dilution of serum that completely inhibits the agglutination. An HI titer ≥ 20 was considered as seropositive and indicated past infection.

Real-time reverse transcription-PCR: Viral RNA was extracted from the oropharyngeal swab samples using the Viral Nucleic Acid Extraction Kit II (Geneaid Biotech Ltd, Taipei, Taiwan) and examined for the influenza virus by real-time RT-PCR. Primer and probe sequences targeting the conserved region of influenza matrix protein, followed the 2009 CDC protocol (CDC protocol). RT-PCR amplification was performed in a DNAEngine® Peltier Thermal Cycler with Chromo4™ Real-Time PCR Detector (Bio-Rad Laboratories Inc, Hercules, CA, USA) using an AccuPower® GreenStar™ RT-qPCR Premix (Bioneer, Daejeon, Korea) with optimized quantitative RT-PCR mixtures

(a 20 µl volume containing 10 µl of 2X master mix, 2 µl of 10X hot star buffer, 0.4 µl of 50X Rox dye, 5 µl of extracted RNA, each 0.8 µM of forward and reverse primers and 0.2 µM of labeled probe and added RNase-free water to bring up the final volume). The amplification cycle was 50°C for 15 mins for reverse

transcription, 95°C for 3 mins for Taq polymerase activation, followed by 45 cycles of PCR amplification (95°C for 15 secs and 55°C for 30 secs). The results were analyzed by MJ OpticonMonitor™ Analysis Software version 3.1 (Bio-Rad).

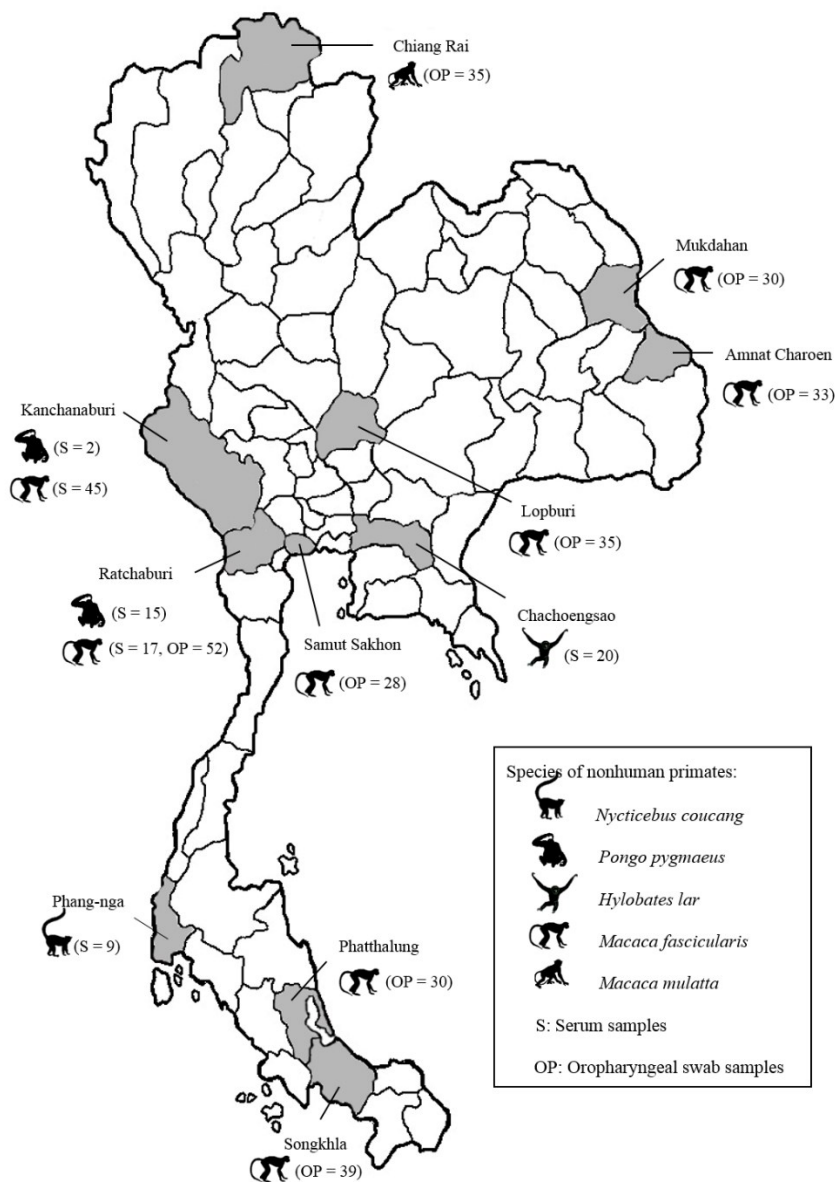


Figure 1 Map of Thailand depicting locations at which serum or oropharyngeal swab samples of the nonhuman primates were collected. The number of samples derived from each species of the nonhuman primates are indicated in parentheses.

Results and Discussion

Our study examined the presence of influenza virus antibody among the 109 archival NHP sera collected between 2009-2017 using ELISA and HI assays. The blocking ELISA assay demonstrated that no influenza A virus-specific NP antibodies were detected in any archival sera. Furthermore, the HI assay showed that all sera had HI titer of <20 against the human H1 virus, LPAI (H1, H3 and H7) and HPAI H5 viruses, indicating that the sera were negative for HI antibodies against the tested influenza viruses. In addition to the influenza virus antibody detection in the NHP serum samples, the study also conducted influenza A viral RNA detection in the newly NHP

oropharyngeal swab samples collected in 2018 using real-time RT-PCR assay. A total of 282 oropharyngeal swab samples was found to be negative for influenza A viral RNA by RT-PCR assay in which the cut-off for positive results was set at 40 cycles. This finding suggested that none of the NHPs were actively infected with influenza virus at the time of sample collection.

The results of the serosurveillance and viral genome detection presented here reveal that NHPs in the study population were not found in infection by and exposure to influenza virus. Conversely, other studies demonstrated that NHPs in South and Southeast Asian countries consisting of Bangladesh, Singapore, Indonesia and Cambodia were found to be naturally infected with avian and human influenza

viruses (Buitendijk *et al.*, 2014; Davis *et al.*, 2015; Karlsson *et al.*, 2012). However, our study had some limitations which have to be pointed out, including lack of the circulating influenza virus strains and an inadequate amount of animal serum used in the serological tests. Further investigation of influenza virus exposure in NHPs should be considered to include currently circulating strains of both human and avian influenza virus for the tested viruses in the serology assays. Moreover, surveillance should be concerned with expanding the sampling population, particularly the NHP population, which has never been monitored in other parts of the country. In Thailand, the NHP population lives close to communities and sometimes shares the same environment with people such as in locations of temples and schools. The close contact between humans and animals possibly contributes to the transmission of influenza virus. Therefore, a continuous monitoring and surveillance program of influenza virus in the NHP population is still necessary.

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