COPYRIGHT NOTICE

FedUni ResearchOnline
https://researchonline.federation.edu.au

This is the peer-reviewed version of the following article:

Suttie, A., et al. (2018). "Influenza A(H5N1) viruses with A(H9N2) single gene (matrix or PB1) reassortment isolated from Cambodian live bird markets." Virology 523: 22-26.

Which has been published in final form at:

https://doi.org/10.1016/j.virol.2018.07.028

Copyright © 2018 Elsevier Inc. All rights reserved.

Title: Influenza A(H5N1) viruses with A(H9N2) single gene (matrix or PB1) reassortment isolated from Cambodian live bird markets

Authors: Annika Suttie[1,2], Erik A Karlsson[1], Yi-Mo Deng[3], Srey Viseth Horm[1], Sokhoun Yann[1], Songha Tok[1], San Sorn[5], Davun Holl[5], Sothyra Tum[4], Aeron C. Hurt[3], Andrew R Greenhill[4], Ian G Barr[3], Paul F Horwood[1,6]*, Philippe Dussart[1]*

* Joint senior authors - these authors contributed equally to the study

Affiliations:

1 - Virology Unit, Institut Pasteur du Cambodge, Institut Pasteur International Network, Phnom Penh, Cambodia

2 - School of Applied and Biomedical Sciences, Federation University, Churchill, Australia

3 - WHO Collaborating Centre for Reference and Research on Influenza, Victorian Infectious Diseases Reference Laboratory, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, 3000, Australia.

4 - National Animal Health and Production Research Institute, Cambodian Ministry of Agriculture, Forestry and Fisheries, Phnom Penh, Cambodia

5 – General Directorate for Animal Health and Production, Cambodian Ministry of Agriculture, Forestry and Fisheries, Phnom Penh, Cambodia

6 - Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, Queensland, 4870, Australia.

Corresponding authors:

Paul Horwood (PhD), Australian Institute of Tropical Health and Medicine

James Cook University, PO Box 6811, Cairns QLD 4870, Australia

Phone: +61 7 4232 1684; Email: paul.horwood@jcu.edu.au

Philippe Dussart (PharmD, PhD), Virology Unit, Institut Pasteur du Cambodge, 5 Monivong Blvd, PO Box 983, Phnom Penh, Cambodia

Phone: +855 23 426 009; Email: pdussart@pasteur-kh.org

Abstract

Live bird market surveillance for avian influenza viruses in Cambodia in 2015 has led to the detection of two 7:1 reassortant influenza A(H5N1) clade 2.3.2.1c viruses. These reassortant strains, designated A/duck/Cambodia/Z564W35M1/2015 and A/chicken/Cambodia/Z850W49M1/2015, both contained a single gene (PB1 and matrix gene, respectively) from concurrently circulating A(H9N2) influenza viruses. All other viral genes from both isolates clustered with A(H5N1) clade 2.3.2.1 viruses. Continued and prolonged co-circulation of influenza A(H5N1) and A(H9N2) viruses in Cambodian live bird markets may present a risk for the emergence of novel influenza reassortant viruses with negative agricultural and/or public health implications.

Keywords

Influenza; A(H5N1); A(H9N2); reassortment; live bird markets; Cambodia

Introduction

Live bird markets (LBMs) in Asia are a critical factor in the persistence of avian influenza viruses in the region and the probable source of many reassortant viruses [1]. The presence of multiple avian species, insufficient biosecurity measures and

constant introduction of immunologically naïve animals all contribute to the heightened risk for the emergence of influenza viruses with pandemic potential. While influenza hemagglutinin subtypes A(H5) and A(H7) are classically considered to have high pandemic potential, several other influenza subtypes including A(H6N1), A(H10N8) and A(H9N2) have crossed the species barrier into humans [2]; and many other avian influenza viruses currently circulate in Asian poultry populations with the potential for adverse animal or human health impacts.

In Cambodia, AIV surveillance in poultry focuses on passive detection of AIVs in reportedly ill animals and active surveillance at LBMs. Cambodian LBMs predominantly sell live chickens and ducks to consumers supplied from backyard flocks. Surveillance for AIVs at the rural farming level is not currently performed. Biosecurity measures at farms and LBMs are either minimal or non-existent. For instance, species separation during the rearing and trading process is not typical and vaccination of poultry against AIVs is illegal. At LBMs overnight bans on holding poultry are in place and veterinary officers that are in charge of each LBM ensure visibly ill poultry are not being traded. Previous studies have shown high levels of AIV prevalence, diversity and AIV subtype co-infections in Cambodian LBMs [1, 3, 4]. In this report we characterize two reassortant A(H5N1) viruses detected during AIV surveillance performed at LBMs in 2015.

Materials and methods

Active surveillance at Cambodian LBMs in 2015 was coordinated by the Institute Pasteur in Cambodia (IPC, WHO National Influenza Centre and H5 Reference Laboratory) and the National Animal Health and Production Research Institute

(NAHPRI, from General Directorate for Animal Health and Production, Cambodian Ministry of Agriculture, Forestry and Fisheries). The sample collection strategy has been described previously [1]. Briefly, environmental and poultry samples were collected weekly from LBMs in Phnom Penh (the capital city of Cambodia) and surrounding areas. Pooled oropharyngeal and cloacal swabs were collected from individual chickens and ducks, randomly selected from each LBM.

Samples were processed according to previously established methods and screened for the presence of influenza A viruses by real-time RT-PCR detection of the matrix protein (MP) gene [1, 3, 4]. Samples that were positive for influenza A virus were further tested for A(H5N1) and other avian influenza viruses of concern (namely H7 and H9) using real-time RT-PCR to detect H5 (primer sets H5a and H5b), N1, H7, and H9 genes. All assays were sourced from the International Reagent Resource (https://www.internationalreagentresource.org/Home.aspx).

To better understand the circulating subtypes in Cambodia, IPC routinely generates full genome avian influenza virus sequences from virus isolates cultured in embryonated eggs, using previously described methods [4, 5]. Briefly, full genome sequences were obtained using the Ion Torrent™ Next Generation Sequencing (NGS) platform at the WHO Collaborating Centre for Reference and Research on Influenza in Melbourne, Australia. Quality control and assembly of NGS data was performed using CLC Genomics Workbench (version 10). Any gaps in NGS sequences were filled using Sanger sequencing. Segment specific primers were used to amplify products that were then sequenced directly with BigDye Terminator v3.1 chemistry using a Genetic Analyzer 3500xL sequencing machine (Life

Technologies). Phylogenetic trees were constructed using the maximum likelihood method based on the general time-reversible model with gamma distribution in RAxML [6]. Bootstrap values were calculated and expressed as a percentage from 1,000 replicates. The GenBank accession numbers for Cambodian strains included in the phylogenetic analysis are: MH285274 - MH285329.

Results and discussion

During the course of isolation and sequencing, we detected two clade 2.3.2.1 influenza A(H5N1) 7:1 reassortant viruses from Cambodian LBM surveillance activities in 2015 that contained single genes from A(H9N2) viruses [1]. These viruses, designated A/duck/Cambodia/Z564W35M1/2015 and A/chicken/Cambodia/Z850W49M1/2015, were originally detected by H5 RT-qPCR from swab material and were subsequently isolated in embyronated chicken eggs. Sequence analysis of the resulting isolates revealed that the viruses arose from two separate reassortment events. The first virus (A/duck/Cambodia/Z564W35M1/2015) was isolated from a duck sample collected in August 2015 and obtained its PB1 gene from A(H9N2). The second virus (A/chicken/Cambodia/Z850W49M1/2015) was isolated from a chicken sample collected in November 2015 and contained a MP gene from an A(H9N2) virus. All other genes from both reassortant viruses originated from influenza A(H5N1) clade 2.3.2.1 (Figure 1/Supplementary Figure 1). Coinfection between A(H5N1) and A(H9N2) in the egg isolates and original samples was ruled out by confirming the absence of an H9 HA gene via PCR analysis.

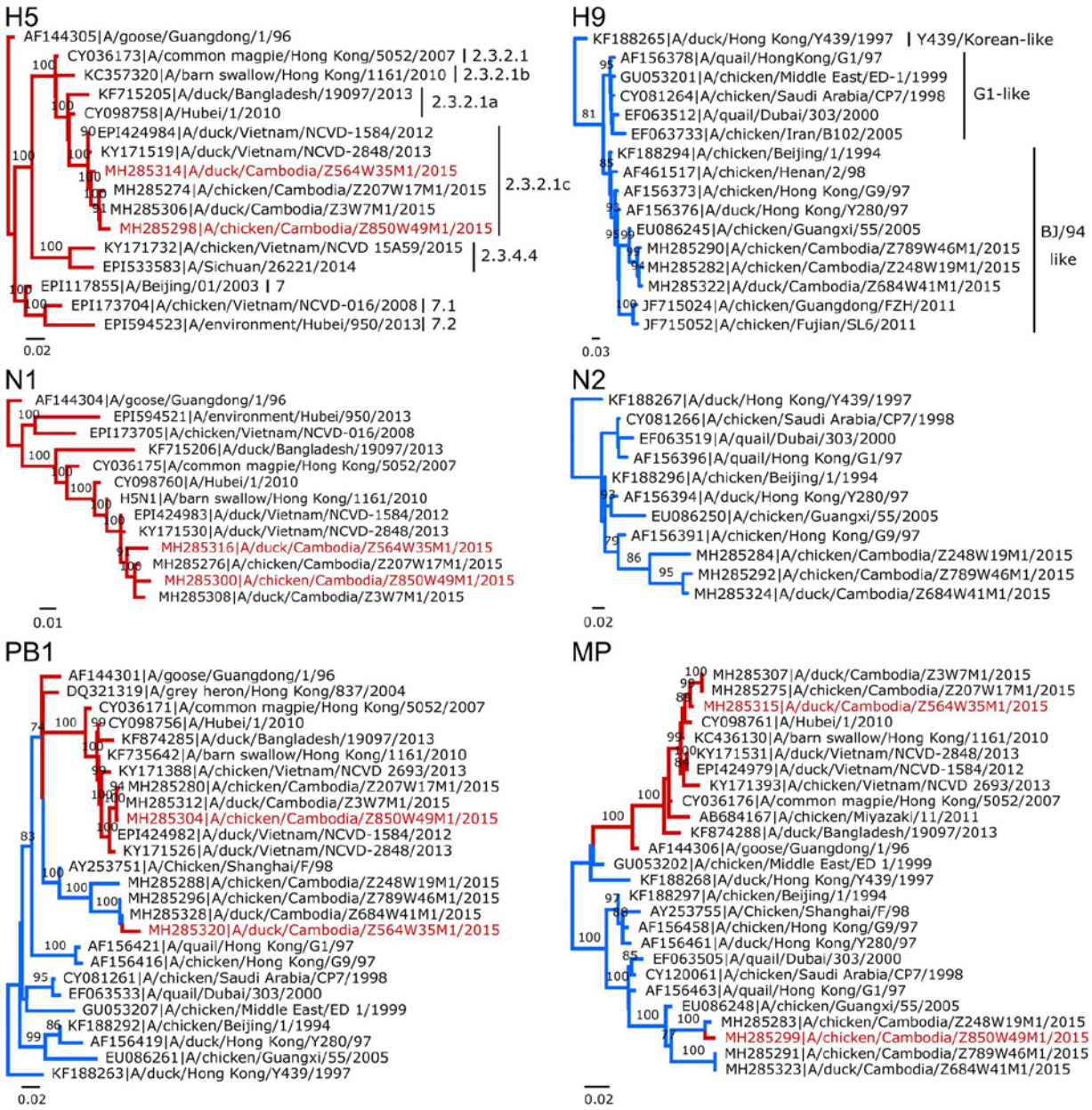

Fig. 1. Phylogenetic analysis of HA, NA, PB1 and MP genomic segments of the Cambodian A(H5N1) reassortant viruses. Trees were constructed using the maximum likelihood method based on the general time-reversible model with gamma distribution. Branches of H5 and N1 genes trees are presented in red. Branches of H9 and N2 genes trees are presented in blue. Branches of PB1 and MP genes are colour coded based on the subtype of HA genes, A(H5) viruses are red and A(H9) viruses are blue. The taxa of both Cambodian A(H5N1) reassortant strains (A/duck/Cambodia/Z564W35M1/2015 and A/chicken/Cambodia/Z850W49M1/2015) have been highlighted in red. Scale bars indicate the number of substitutions per site and bootstrap values (n = 1000) of 70 or greater are shown.

The replacement of the A(H5N1) MP gene with that from A(H9N2) in strain A/chicken/Cambodia/Z850W49M1/2015 resulted in the acquisition adamantane drug resistance due to the presence of the M2 amino acid substitution S31N. Homotypic A(H5N1) clade 2.3.2.1 viruses are generally susceptible to adamantanes as they do not carry any of the genetic markers of adamantane resistance. Out of 808 A(H5) clade 2.3.2.1 viruses (including subclades 2.3.2.1a-2.3.2.1c) found on the Influenza Research Database (https://www.fludb.org), only 5% contained the S31N M2 mutation [7].

Influenza A(H9N2) is a low pathogenic avian influenza virus that has become widespread throughout Asia and Africa since its first appearance in Southern China in 1994. Often co-circulating with other subtypes of concern, the A(H9N2) subtype has been detected in humans and pigs, so is considered a pandemic threat [2]. In addition, A(H9N2) viruses appear to be extremely promiscuous and can easily reassort with other influenza viruses, often producing novel strains with increased ability to jump the species gap. Prior to the emergence of A(H7N9) in China, endemic chicken A(H9N2) viruses provided all of the internal genes through numerous reassortment events [8]. Indeed, due to the ongoing co-circulation of these viruses, further reassortments between A(H7N9) and A(H9N2) are frequently reported [9]. Additionally, influenza A(H9N2) contributed all of the internal genes to an A(H10N8) virus that caused a fatal infection in China in 2013 [10].

Interestingly, despite widespread, sustained co-circulation and frequent coinfection of influenza A(H5Nx) and A(H9N2), natural reassortants between these viruses are seldom detected (Table 1). Similar to the viruses described here from Cambodia,

other reassortant viruses with an A(H5N1) backbone and single gene reassortment (7:1) with an A(H9N2) MP or PB1 gene have previously been identified in Bangladesh [11–13]. There are also reports of A(H5N1) viruses with PB2 genes from A(H9N2) from Africa, India and the United Arab Emirates [14–16]. Further, reassortant viruses with an A(H9N2) backbone and single gene reassortment with A(H5N1) have been described, including: viruses in Pakistan with an A(H5N1) NS gene; viruses in Southern China with an A(H5N1) PB1 gene; and viruses in Eastern China with an A(H5N1) HA gene [17–19].

There are even fewer reports of multiple gene segment A(H5N1)/A(H9N2) reassortant viruses. Phylogenetic studies have revealed that influenza A(H9N2) may have provided the internal genes prior to the emergence of A(H5N1) in Hong Kong, 1997 [20]. More recently, A(H5N2) viruses were detected in chickens from Tibet and Vietnam with half of the gene fragments originating from A(H5N1) and the other genes from A(H9N2) [21, 22]. There was also a report of an A(H5N2) virus from China where all viral genes aside from HA and M clustered with A(H9N2) [23]. Evidence of human infection with these reassortant viruses is extremely rare but they have been detected previously. In 2013, a Canadian resident died following a respiratory illness with meningoencephalitis after recently returning from China. The cause of the illness was confirmed to be influenza a clade 2.3.2.1c A(H5N1) 7:1 reassortant virus with an A(H9N2) PB2 gene [24].

Through active and passive surveillance systems, IPC and NAHPRI have documented high levels of avian influenza circulation in Cambodian LBMs, with co-circulation of multiple viral subtypes [1, 3, 4]. During the 2015 LBM study 45% (342/752) of the poultry samples collected were influenza A positive. High rates of

A(H5N1) and A(H9N2) coinfections were also detected in poultry, with both viruses identified in 4.5% of chickens and 0.8% of ducks [1]. There is also evidence of subclinical human infections with these viruses in Cambodian LBMs, with antibodies to A(H5N1) and A(H9N2) detected in 4.5% and 1.8% of poultry workers, respectively [4]. High co-circulation of a variety of avian influenza viruses is a risk for the emergence of novel reassortant influenza viruses, as was exemplified by a novel A(H5N1) clade 1.1.2 virus reassortant that emerged in poultry in Cambodia and Vietnam in 2013-2014. This virus contained the HA/NA genes from a clade 1.1.2 genotype Z virus and the internal and matrix genes from a clade 2.3.2.1a virus [5, 25]. This novel reassortant caused outbreaks in poultry as well as several human cases in both countries. In Cambodia the emergence of this virus was associated with a dramatic increase in human cases during 2013 and early 2014 [26].

Little is known about the impact of single A(H9N2) gene (7:1) mutants on the fitness or pathogenicity of A(H5) viruses. Further *in vitro* and *in vivo* experiments are necessary to understand how the contribution of A(H9N2) genes could affect A(H5N1) pathogenicity in chickens and if there is an increased risk to human health. It is possible that these reassortant viruses may instead have lowered fitness in poultry populations and be unable to outcompete currently circulating wildtype A(H5N1) viruses, as was suggested for similar reassortant viruses in Bangladesh [11]. This lack of fitness is also postulated for these Cambodian strains, as no other reassortants have been detected to date since avian influenza surveillance started in 2011.

Conclusions

Overall, the detection of multiple A(H5N1)/A(H9N2) (7:1) viruses exemplifies that further surveillance is necessary in Cambodia and other countries with endemic circulation of avian influenza viruses. Indeed, continual and vigilant surveillance is absolutely crucial in areas endemic for influenza subtypes known to be a public health risk, such as A(H5Nx), A(H7Nx) and A(H9N2). In addition, interventions are urgently needed to reduce the risk of the emergence of novel avian influenza viruses that could constitute a pandemic threat.

Acknowledgements

The authors thank the field and laboratory teams at the Institute Pasteur in Cambodia and the National Animal Health and Production Research Institute for their efforts. We also thank the vendors from the participating live bird markets for their cooperation with our study team. This publication is the result of work conducted under a cooperative agreement with the Office of the Assistant Secretary for Preparedness and Response in the U.S. Department of Health and Human Services (HHS), grant number IDSEP140020-01-00. Its contents and conclusions are solely the responsibility of the authors and do not represent the official views of HHS. The study was also funded, in part, by the US Agency for International Development (grant No. AID-442-G-14-00005). Annika Suttie is funded by an Australian Government Research Training Program Scholarship and a Faculty of Science and Technology Research Scholarship from Federation University. The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health.

Competing interests

The authors declare that they have no competing interests.

References

1. Horwood PF, Horm SV, Suttie A, Thet S, Phalla Y, Rith S, et al. Co-circulation of influenza A H5, H7, and H9 viruses and co-infected poultry in live bird markets, Cambodia. Emerging Infectious Diseases. 2018;24:352–5.

2. Burke SA, Trock SC. Use of Influenza Risk Assessment Tool for Prepandemic Preparedness. Emerging infectious diseases. 2018;24:471.

3. Horm SV, Sorn S, Allal L, Buchy P. Influenza A(H5N1) Virus Surveillance at Live Poultry Markets, Cambodia, 2011. Emerging Infectious Diseases. 2013;19:305–8.

4. Horm SV, Tarantola A, Rith S, Ly S, Gambaretti J, Duong V, et al. Intense circulation of A/H5N1 and other avian influenza viruses in Cambodian live-bird markets with serological evidence of sub-clinical human infections. Emerging Microbes & Infections. 2016;5:e70.

5. Rith S, Davis CT, Duong V, Sar B, Horm SV, Chin S, et al. Identification of Molecular Markers Associated with Alteration of Receptor-Binding Specificity in a Novel Genotype of Highly Pathogenic Avian Influenza A(H5N1) Viruses Detected in Cambodia in 2013. Journal of Virology. 2014;88:13897–909.

6. Stamatakis A. RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. Bioinformatics. 2014;30:1312–1313.

7. Squires RB, Noronha J, Hunt V, García-Sastre A, Macken C, Baumgarth N, et al. Influenza Research Database: an integrated bioinformatics resource for influenza

research and surveillance: Influenza Research Database. Influenza and Other Respiratory Viruses. 2012;6:404–16.

8. Pu J, Wang S, Yin Y, Zhang G, Carter RA, Wang J, et al. Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. Proceedings of the National Academy of Sciences. 2015;112:548–53.

9. Cui L, Liu D, Shi W, Pan J, Qi X, Li X, et al. Dynamic reassortments and genetic heterogeneity of the human-infecting influenza A (H7N9) virus. Nature communications. 2014;5:3142.

10. Chen H, Yuan H, Gao R, Zhang J, Wang D, Xiong Y, et al. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. The Lancet. 2014;383:714–721.

11. Marinova-Petkova A, Feeroz MM, Rabiul Alam S, Kamrul Hasan M, Akhtar S, Jones-Engel L, et al. Multiple introductions of highly pathogenic avian influenza H5N1 viruses into Bangladesh. Emerging Microbes & Infections. 2014;3:e11.

12. Monne I, Yamage M, Dauphin G, Claes F, Ahmed G, Giasuddin M, et al. Reassortant Avian Influenza A(H5N1) Viruses with H9N2-PB1 Gene in Poultry, Bangladesh. Emerging Infectious Diseases. 2013;19:1630–4.

13. Gerloff NA, Khan SU, Balish A, Shanta IS, Simpson N, Berman L, et al. Multiple reassortment events among highly pathogenic avian influenza A(H5N1) viruses detected in Bangladesh. Virology. 2014;450–451:297–307.

14. Tosh C, Nagarajan S, Kumar M, Murugkar HV, Venkatesh G, Shukla S, et al. Multiple introductions of a reassortant H5N1 avian influenza virus of clade 2.3.2.1c

with PB2 gene of H9N2 subtype into Indian poultry. Infection, Genetics and Evolution. 2016;43:173–8.

15. Naguib MM, Kinne J, Chen H, Chan K-H, Joseph S, Wong P-C, et al. Outbreaks of highly pathogenic avian influenza H5N1 clade 2.3. 2.1 c in hunting falcons and kept wild birds in Dubai implicate intercontinental virus spread. Journal of General Virology. 2015;96:3212–3222.

16. Tassoni L, Fusaro A, Milani A, Lemey P, Awuni JA, Sedor VB, et al. Genetically Different Highly Pathogenic Avian Influenza A(H5N1) Viruses in West Africa, 2015. Emerging Infectious Diseases. 2016;22:2132–6.

17. Iqbal M, Yaqub T, Reddy K, McCauley JW. Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. PloS one. 2009;4:e5788.

18. Dong G, Xu C, Wang C, Wu B, Luo J, Zhang H, et al. Reassortant H9N2 influenza viruses containing H5N1-like PB1 genes isolated from black-billed magpies in Southern China. PloS one. 2011;6:e25808.

19. Zhao G, Gu X, Lu X, Pan J, Duan Z, Zhao K, et al. Novel Reassortant Highly Pathogenic H5N2 Avian Influenza Viruses in Poultry in China. PLoS ONE. 2012;7:e46183.

20. Guan Y, Shortridge KF, Krauss S, Webster RG. Molecular characterization of H9N2 influenza viruses: were they the donors of the “internal” genes of H5N1 viruses in Hong Kong? Proceedings of the National Academy of Sciences. 1999;96:9363–9367.

21. Zhao S, Suo L, Jin M. Genetic characterization of a novel recombinant H5N2 avian influenza virus isolated from chickens in Tibet. Journal of virology. 2012;86:13836–13837.

22. Nishi T, Okamatsu M, Sakurai K, CHU HD, Thanh LP, van NGUYEN L, et al. Genetic analysis of an H5N2 highly pathogenic avian influenza virus isolated from a chicken in a live bird market in Northern Vietnam in 2012. Journal of Veterinary Medical Science. 2014;76:85–87.

23. Xu H, Meng F, Huang D, Sheng X, Wang Y, Zhang W, et al. Genomic and Phylogenetic Characterization of Novel, Recombinant H5N2 Avian Influenza Virus Strains Isolated from Vaccinated Chickens with Clinical Symptoms in China. Viruses. 2015;7:887–98.

24. Pabbaraju K, Tellier R, Wong S, Li Y, Bastien N, Tang JW, et al. Full-genome analysis of avian influenza A (H5N1) virus from a human, North America, 2013. Emerging infectious diseases. 2014;20:887.

25. Thor SW, Nguyen H, Balish A, Hoang AN, Gustin KM, Nhung PT, et al. Detection and characterization of Clade 1 Reassortant H5N1 viruses Isolated from human cases in Vietnam during 2013. PloS one. 2015;10:e0133867.

26. Ly S, Horwood P, Chan M, Rith S, Sorn S, Oeung K, et al. Seroprevalence and Transmission of Human Influenza A(H5N1) Virus before and after Virus Reassortment, Cambodia, 2006–2014. Emerging Infectious Diseases. 2017;23:300–3.

Tables and Figures

Table 1. Natural influenza A(H5N1) and A(H9N2) reassortant viruses reported in the literature

Figure 1. Phylogenetic analysis of HA, NA, PB1 and MP genomic segments of the Cambodian A(H5N1) reassortant viruses. Trees were constructed using the maximum likelihood method based on the general time-reversible model with gamma distribution. Branches of H5 and N1 genes trees are presented in red. Branches of H9 and N2 genes trees are presented in blue. Branches of PB1 and MP genes are colour coded based on the subtype of HA genes, A(H5) viruses are red and A(H9) viruses are blue. The taxa of both Cambodian A(H5N1) reassortant strains (A/duck/Cambodia/Z564W35M1/2015 and A/chicken/Cambodia/Z850W49M1/2015) have been highlighted in red. Scale bars indicate the number of substitutions per site and bootstrap values (n=1,000) of 70 or greater are shown.

Supplementary Figure 1. Phylogenetic analysis of PB2, PA, NP and NS genomic segments of the Cambodian A(H5N1) reassortant viruses. Trees were constructed using the maximum likelihood method based on the general time-reversible model with gamma distribution. Branches are colour coded based on the subtype of HA genes, A(H5) viruses are red and A(H9) viruses are blue. The taxa of both Cambodian A(H5N1) reassortant strains (A/duck/Cambodia/Z564W35M1/2015 and A/chicken/Cambodia/Z850W49M1/2015) have been highlighted in red. Scale bars indicate the number of substitutions per site and bootstrap values (n=1,000) of 70 or greater are shown.