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Short Article

Bat cells from *Pteropus alecto* are susceptible to influenza A virus infection and reassortment

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Waterfowl are primary hosts for influenza A viruses (IAVs); however, there is sporadic infection of swine and other species that pose a risk of zoonotic spread. Yellow-shouldered bats were shown to be hosts of an IAV, thereby constituting a potential novel reservoir. We show that *Pteropus alecto* kidney cells (PaKi) are susceptible to infection and sustain replication of A/WSN/33 (H1N1) and A/Vietnam/1203/04 (H5N1). Importantly, we show that co-infection of PaKi cells results in novel reassortants.

Keywords Bats, H1N1, H5N1, influenza, reassortment.

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Introduction

Numerous species are susceptible to infection with influenza A viruses (IAV) including wild and domesticated avian species, horses, dogs, ferrets, swine, whales, and seals.[1] IAVs often cause asymptomatic infections, particularly in wildfowl and shorebirds,[1] and additional species are known to serve as intermediate hosts, which may provide a niche in the evolution and ecology of IAVs. Bats are a species of interest contributing to approximately 25% of all known mammalian species, and bats have emerged as a primary reservoir for a variety of zoonotic diseases spanning numerous virus families including the *Paramyxoviridae*, *Rhabdoviridae*, *Coronavir*idae, and *Filovir*idae.[2–4] Bats of the genus Pteropus (flying foxes) were first identified as a reservoir of Hendra virus.[5,6] Bats have also been identified as reservoirs for other zoonotic diseases such as SARS-COV and Ebola virus. Previously, in the 1970s, influenza hemagglutinin and neuraminidase proteins were isolated from the lungs and trachea of *Nyctalus noctula* bats.[7] Recently, a new lineage of IAV was identified from genetic material isolated from rectal swabs of bats indigenous to Guatemala, revealing novel HA and NA subtypes.[8] To date, efforts to isolate and grow this newly identified virus have been unsuccessful. However, the identification of bats as a potential reservoir for IAV is important to understand particularly as related to host–pathogen interactions that include replication, transmission, and adaptation.

In this study, the susceptibility of *Pteropus alecto* kidney (PaKi) cells[9] to infection with IAVs was investigated. Initially, a human strain of IAV, that is, A/WSN/33 (H1N1), was investigated for its ability to infect PaKi cells. Cells were infected with A/WSN/33 H1N1 at an MOI of 0·01 in DMEM supplemented with 5% fetal bovine serum (FBS) without the addition of exogenous trypsin for 24 hours at 37°C. Infected cells were then examined by immunofluorescence using a mouse anti-NP primary antibody and a goat anti-mouse Alexa Flour 488-conjugated secondary antibody and visualized using confocal microscopy. As shown in Figure 1A, NP staining was observed in the nucleus and cytoplasm of PaKi cells indicating that bat cells are susceptible to infection with IAV.

Next, the replication kinetics of several IAV strains including A/WSN/33 (H1N1), A/California/04/09 (H1N1), A/Vietnam/1203/04 (H5N1), and A/Swine/Illinois/02860/08 (H1N1) were examined in PaKi cells infected at a MOI of 0·01 at 37°C at 24 and 48 hpi by $TCID_{50}$ titers. Attempts were made to determine the replication kinetics of A/California/04/09 and A/Swine/Illinois/02860/08, but due to exogenous trypsin sensitivity of PaKi cells resulting in loss of the cell monolayer, this was not possible. However, these viruses were able to infect PaKi cells as evidenced by NP staining at 12 hpi (data not shown). Because the HA of A/Vietnam/1203/2004 contains a polybasic cleavage site allowing for cleavage by an endogenous furin-like protease [10] and the HA of A/WSN/33 is cleaved by the serum protease plasmin, the

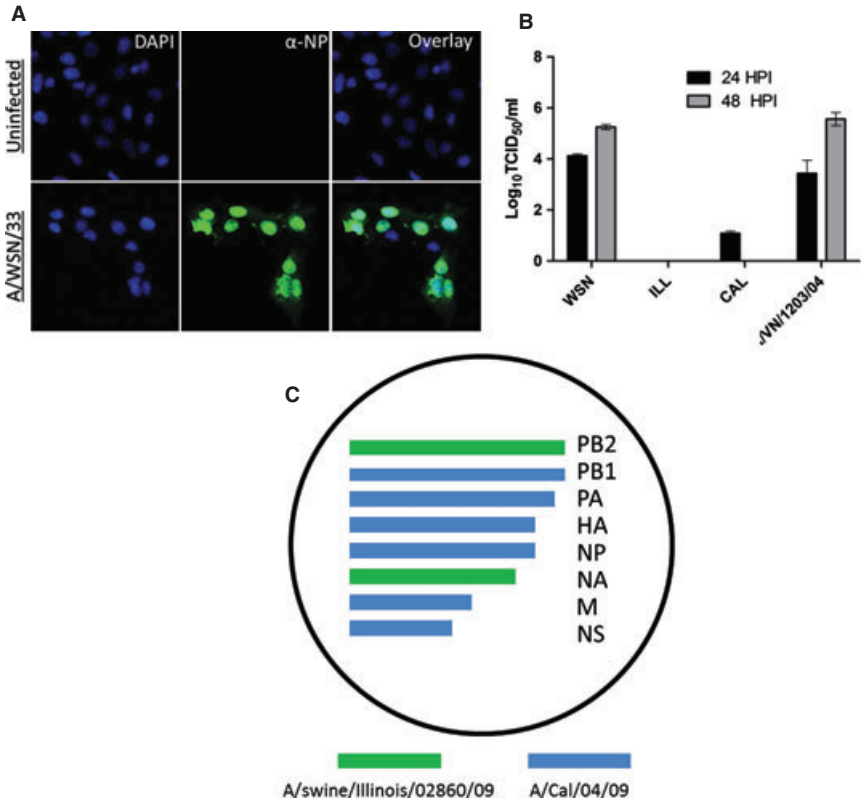

Figure 1. Susceptibility of bat epithelial cells to infection with multiple strains of IAV and the propensity for co-infection and development of a novel virus. (A). PaKi cells were infected with A/WSN/33 at an MOI 0·01 or mock infected with media. Cells were fixed 12 hpi with 2% formalin and stained using a mouse anti-NP primary antibody and goat anti-mouse Alexa Flour 488 secondary antibodies. Cells were visualized using confocal microscopy. (B) PaKi cells were infected with A/WSN/33 and A/VN/1203/04 supplemented with 5% fetal bovines serum and A/swine/Illinois/02860/09 (ILL) and A/California/04/09 (Cal) supplemented with 1 ug/ml TPCK-treated trypsin at a 0·01 of MOI. TCID50 assays were conducted on cell supernatants isolated 24 and 48 hpi. Data are presented as the average of three independent experiments performed in duplicate. (C) PaKi cells were co-infected with A/Swine/Illinois/02860/09 and A/California/04/09 with a MOI of 3·0. Cell supernatants were collected and evaluated for the presence of reassortant viruses. Shown is a schematic of the single reassortant isolated post-co-infection of PaKi cells. The viral isolate was confirmed by Sanger sequencing.

addition of exogenous trypsin was not necessary[11]; thus, the replication kinetics were determined. For A/WSN/33 and A/Vietnam/1203/2004, the inoculum was supplemented with 5% FBS and 0·5% L-Gln, respectively. As shown in Figure 1B, A/WSN/33 virus replicated efficiently at 24 and 48 hpi with titers of $10^{4.1}$ and $10^{5.25}$ TCID50/ml, respectively. Similarly, the A/Vietnam/1203/04 virus also replicated efficiently, with titers of $10^{4.1}$ and $10^{5.6}$ TCID50/ml at 24 and 48 hpi, respectively. Infection with A/Vietnam/1203/04 induces substantial cytopathic effect (CPE) 48 hpi with almost full disruption of the cell monolayer. Infection with A/WSN/33 induced a minimal amount of CPE visible at 48 hpi. These findings show that PaKi cells not only are capable of supporting IAV infection, but also sustain IAV replication.

From these findings, the propensity for the generation of reassortant viruses was examined following co-infection of PaKi cells with A/California/04/09 and A/Swine/Illinois/02860/08 at an MOI of 3 per virus in DMEM supplemented with 0·5% L-glutamine. Because these viruses require the presence of trypsin for multiple rounds of replication, infections were limited to one round of replication. Supernatants were collected at 24 hpi and plaqued on MDCK cells. Individual plaques were picked and amplified in MDCK cells. Supernatants were collected at 72 hpi, and RNA was isolated using the RNeasy kit (Qiagen). cDNA was synthesized from the purified RNA using the Verso cDNA synthesis kit (Thermo Scientific). The residual RNA was precipitated as described previously,[12] and cDNAs were further purified using the QiaQuick PCR Purification kit (Qiagen). cDNA from individual plaques was then screened using triplex TaqMan qPCR specific for each virus with primers and probes specifically targeting the HA, NA, and PB2 gene segments (Table 1). Reactions were performed using the Quantifast Multiplex qPCR kit (Qiagen).

A total of 77 plaques were screened and two reassortants identified. Of the two reassortants, positive Ct values ranging from 24 to 35 were observed for the A/Swine/Illinois/02860/08 NA and the A/California/04/09 HA and PB2 genes, and

Table 1. Reassortant screen multiplex primers and probes

	Forward Primer	Reverse Primer	Probe
ILL HA	GGTGTTCATCATCCGCCTAACAT	GCCTCTACTCAGTGTGAAAGCATACCAT	CAACTACTACTGGACTCTGCTGGAACC
ILL NA	TGTGCATAGCATGGTCCAGCTCAA	AAAGGATATGCTGCTCCCGCTAGT	AGGGCGACCCAAAGAGAACACAAT
ILLPB2	TGGCCCAGTCCACTTCAGAAATCA	ATAAACACTGCCTGTTCCACCAGC	GACGAGGTTTCTCCCGGTGGCTGGT
CAL HA	CACTCTCCACAGCAAGCTCA	CTTCTTGCTGTATCTTGATGACC	ATCACTCTCCACAGCAAGCTCAT
CAL NA	TGATTTGGGATCCGAACGGATGGA	AAAGGATATGCTGCTCCCGCTAGT	AGGGCGACCCAAAGAGAACACAAT
CAL PB2	TGACATCAGAGTCACAGCTGGCAA	TCTGCTGACACTGCTGCTCTTCTT	AAACAAGGTTTCTCCCAGTAGCCGGCG

the other isolate exhibited positive Ct values for the A/Swine/Illinois/02860/09 HA and A/California/04/09 NA and PB2 genes (Figure 1C). The reassortants were subjected to full-genome sequencing. One-step RT-PCR was performed on viral RNA using virus-specific amplification primers. Amplified products were gel-purified using the QIAquick gel purification kit (Qiagen) according to manufactures' protocol. Full-genome sequencing using primers encompassing all 8 genes from A/California/04/09 and A/Swine/Illinois/02860/09 was used. Sequencing reactions were carried out with the Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and sequenced on an ABI 3130-XL Genetic Analyzer. Sequencing verified that one isolate was a reassortant viruses resulting from co-infection of PaKi cells (Figure 1C). The identification of a two reassortants prior to sequencing probably resulted from non-specific cross-reactivity of the multiplex screen. Collectively, these studies provide the first demonstration that bat cells can sustain replication of IAV and support the generation of influenza virus reassortants.

As shown, bat epithelial cells like swine cells are susceptible to infection by both avian and human IAV strains. Swine have served as a reservoir for the past three pandemics including the 2009 pandemic that resulted from a triple reassortant virus derived from human, swine, and avian lineages.[13,14] Importantly, given the results presented here, additional studies are warranted that aim to determine the role of bats in the ecology of IAV reassortment and the identification of additional mixing vessels for the generation of reassortant IAV isolates. It has been identified that bats have complex direct and indirect interactions with multiple reservoirs of IAV. Interactions have been observed in urban Hong Kong in which local fruit bats and bird species fed on flowering plants in the same locale.[15] These distinct interactions present an opportunity for transmission of IAV from birds to bats. With HPAI actively circulating in avian species in China,[16] the identification of a novel HA and NA subtype in bats[8] and initial evidence for support of HPAI replication in bat epithelial cells establish the need for additional studies investigating the potential of bats as a reservoir for IAV, the host–pathogen interactions between bats and IAV, and the need for increased surveillance in bat populations.

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