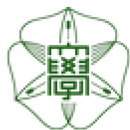
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Generation of bat-derived influenza viruses and detection of virus-specific antibody in African bats
（コウモリ由来インフルエンザウイルスの作出とアフリカのコウモリからの特異抗体検出）

Masahiro SATO

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Abbreviation

Aa	Amino acid
BatIV	bat-derived influenza virus
BLAST	basic local alignment search tool
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
HA	hemagglutinin
HEK	human embryonic kidney
HL	HA-like
HRP	horseradish peroxidase
IAV	influenza A virus
IFA	immunofluorescent assay
IgG	immunoglobulin G
IU	infectious units
M1	matrix protein
MDCK	Madin-Darby canine kidney
MERS	middle east respiratory syndrome
NA	neuraminidase
NL	NA-like
NP	nucleoprotein
OD	optical density
PBS	phosphate-buffered saline
PBST	0.05% Tween 20 in PBS

PCR	polymerase chain reaction
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
SARS	severe acute respiratory syndrome
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
TMB	3,3′,5,5′-Tetramethylbenzidine
TPB	tryptose phosphate broth
VSV	vesicular stomatitis virus

Notes

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1. Sato, M., Maruyama, J., Kondoh, T., Nao, N., Miyamoto, H., Takadate, Y., Furuyama, W., Kajihara, M., Ogawa, H., Manzoor, R., Yoshida, R., Igarashi, M. & Takada, A. Generation of bat-derived influenza viruses and their reassortants. *Sci Rep*. In press.

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Preface

Influenza A viruses (IAVs), which belong to the family *Orthomyxoviridae*, cause highly contagious diseases in a wide variety of avian and mammalian species, including humans, pigs, horses, dogs, and poultry, and are recognized as one of the most important zoonotic pathogens. IAVs have 8 segmented negative-sense ribonucleic acid (RNA) genomes and are divided into subtypes based on combination of two viral envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). IAVs with H1-16 HA and N1-9 NA subtypes have been identified in wild aquatic birds, especially migratory ducks, which are believed to be the natural reservoir of IAVs[1,2,3,4]. Importantly, due to their propensity for genetic reassortment, a variety of IAV subtypes are distributed in many host species.

Recently, influenza virus-like RNA genomes were detected in fruit bats (*Sturnira lilium* and *Artibeus planirostris*) in Central and South America, respectively[5,6]. Since the amino acid sequences of their HAs and NAs are distinct from those of all previously known IAV subtypes, these bat-derived influenza viruses (BatIVs) have been provisionally designated H17N10 and H18N11. However, previous studies have reported that H17 HA does not bind to sialic acids linked to galactose in sugar chains[7], which are known as canonical receptors of IAVs, and that N10 NA lacks neuraminidase activity[8] which is also a common property of all known IAV NAs. Thus, BatIV glycoproteins have also been called HA-like (HL) and NA-like (NL) (i.e., HL17NL10 and HL18NL11)[9]. However, information on the biological properties of these BatIVs is limited since infectious virus particles have never been isolated from infected animals.

Bats belonging to the order Chiroptera, which is known as the second largest order of mammalians, are distributed into more than 1,000 species globally[10]. It has

been shown that bats play crucial roles as natural reservoirs of some zoonotic pathogens such as Marburg virus, Hendra virus, Nipah virus, Lyssa virus, severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS) coronaviruses[11,12,13,14]. In addition to the above mentioned H17N10 and H18N11 BatIVs, avian H9N2 IAV-like BatIV strains were recently isolated from a frugivorous bat species in Egypt, providing a new insight into influenza virus ecology and evolution in nature[15]. These accumulated evidences suggest the importance to investigate the ecology of bat-derived pathogens and to clarify their potential risks as zoonotic pathogens.

Biological properties (e.g. replication cycles, receptor molecules, and host range) and ecological distribution of BatIVs are still largely unknown due to the limited number of available reports. Therefore, infectious BatIVs and their reassortants were generated to confirm their infectivity in the bat cell lines which have been shown to be susceptible to BatIV HA-pseudotyped virus[16]. In addition, serological prevalence of IAV infection in bats captured in Zambia was investigated to obtain basic information on the distribution of BatIVs in the African continent.

Chapter I:

Generation of bat-derived influenza viruses and their reassortants

Introduction

Novel BatIVs (i.e. H17N10 and H18N11) were detected in frugivorous bats (*Sturnira lilium* and *Artibeus planirostris*) in Guatemala and Peru, respectively[5,6]. Since BatIV glycoproteins (e.g. H17 HA and N11 NA) have different biological properties from those of all known IAVs, these BatIV glycoproteins have also been called HA-like (HL) and NA-like (NL) proteins (i.e., HL17NL10 and HL18NL11)[9]. The biological properties of BatIVs were largely unknown since infectious virus particles have never been isolated from host animals.

The previous study revealed that vesicular stomatitis viruses (VSVs) pseudotyped with BatIV glycoproteins efficiently infected cultured cells derived from particular bat species (e.g. *Miniopterus fuliginosus*) but not those commonly used for IAV propagation and other bat cells tested, providing key information on cell lines that are potentially susceptible to BatIVs[16]. Using these bat cell lines and the well-known plasmid-based reverse genetics approach[17], the generation of infectious BatIVs and their reassortants were demonstrated in this study.

Materials and Methods

Cells. HEK293T and Vero E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, 10% FCS, 100 U/ml penicillin, and 0.1mg/ml streptomycin. Madin-Darby canine kidney (MDCK) cells were grown in DMEM supplemented with L-glutamine, 10% calf serum, and penicillin-streptomycin. QT6 cells were grown in F-12K medium supplemented with 5% FCS, 5% tryptose

phosphate broth (TPB), and penicillin-streptomycin, MDCK II cells were grown in Eagle's minimum essential medium supplemented with L-glutamine, 5% FCS. All bat-derived cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with L-glutamine, 10% FCS, and penicillin-streptomycin[18,19].

Construction of plasmids. All segments of H17N10 and H18N11 BatIV genes (GenBank Accession numbers CY103881-CY103889 and CY125942-CY125949) were amplified using plasmids encoding each gene segment (kindly provided from Dr. Suxiang Tong) and cloned into the Pol-I plasmid pHH21 as described previously[17], using Gibson Assembly Master Mix (New England Bio Lab). Viral polymerase (i.e., PB2, PB1, and PA) and NP genes were cloned into the protein expression vector pCAGGS[20].

Generation of BatIVs and their reassortants. HEK293T cells (4.0×10^5) were seeded into 6-well plates and transfected with eight Pol-I plasmids (0.1 μg for each segment) and four protein expression plasmids (1.0 μg for PB2, PB1, PA, and NP) using TransIT-LT1 (Mirus Bio LLC) according to the manufacturer's protocol. Supernatants of the transfected cells were harvested at 48 hours post-transfection and were centrifuged through a 25% sucrose cushion (133,900 x g, 2 hours, 4°C). Concentrated (200X) virus particles were treated with 5.0 μg/ml trypsin for 1 hour at 37°C and then inoculated into YubFKT2 and other cells seeded on 6-well plates.

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were conducted as described previously[15]. Briefly,

concentrated BatIV particles were mixed with SDS-PAGE sample buffer with 5% 2-mercaptoethanol and boiled for 5 minutes. After electrophoresis on 5–20% SuperSep Ace (Wako), separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with a mouse anti-M1 monoclonal antibody (APH 6-23-1-6)[21], a mouse anti-HA2 monoclonal antibody produced in our laboratory (H13N6 148-6-6) or anti-N10 NA rabbit polyclonal antibody (FS0181) recognizing amino acid positions 328-343 (AQEKGEGGIQGFILDE) followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (H+L) or goat anti-mouse IgG (H+L) (Jackson ImmunoResearch). The bound antibodies were visualized with Immobilon Western (Millipore).

Transmission electron microscopy (TEM). BatIV particles fixed with 0.25% glutaraldehyde were adsorbed onto collodion-carbon-coated copper grids and negatively stained with 2% phosphotungstic acid solution (pH 5.8). For immuno-TEM, I used an anti-HA2 mouse monoclonal antibody (H13N6 148-6-6) and anti-N10 NA rabbit polyclonal antibody (FS0181) as primary antibodies and immunogold-conjugated goat anti-mouse IgG (H+L) 5 nm Gold (BB International) and goat anti-rabbit IgG (H + L) 15 nm Gold (Abcam) antibodies. Samples were examined with an H-7650 electron microscope (Hitachi) at 80 kV.

Immunofluorescent assay. Forty-eight hours after inoculation of BatIV, the cells were fixed with 4% formalin in phosphate-buffered saline (PBS) for 20 minutes. They were then blocked with 2% bovine serum albumin/PBS overnight at 4°C and then permeabilized for 7.5 minutes using 0.2% Triton X-100/PBS. The cells were stained

with anti-M1 (APH 6-23-1-6) or anti-HA2 (H13N6 148-6-6) monoclonal antibodies and an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen). Samples were observed with an LSM780 confocal microscope (Carl Zeiss).

Results

Generation of BatIV from plasmids. First, generation of H17N10 BatIV was conducted by transfecting human embryonic kidney (HEK) 293T cells with 8 pol-I plasmids providing viral RNA templates and 4 pCAGGS plasmids expressing the viral nucleoprotein (NP) and polymerases required for viral RNA transcription/replication. To confirm the expression of HA, NA, and viral matrix protein (M1) in the transfected cells, cell lysates and supernatants were analyzed by western blotting. H17 HA, N10 NA, and M1 were detected as approximately 70 kDa, 70 kDa, and 27 kDa bands, respectively, in the cell lysates (Figure 1a), suggesting that plasmid-driven viral RNA transcription/replication successfully occurred in the transfected cells. Furthermore, these viral proteins were detectable in the supernatants of the transfected cells (Figure 1b). Pol-I and pCAGGS plasmids providing viral RNA and proteins of H18N11 BatIV were also constructed and similarly used for generation of the virus.

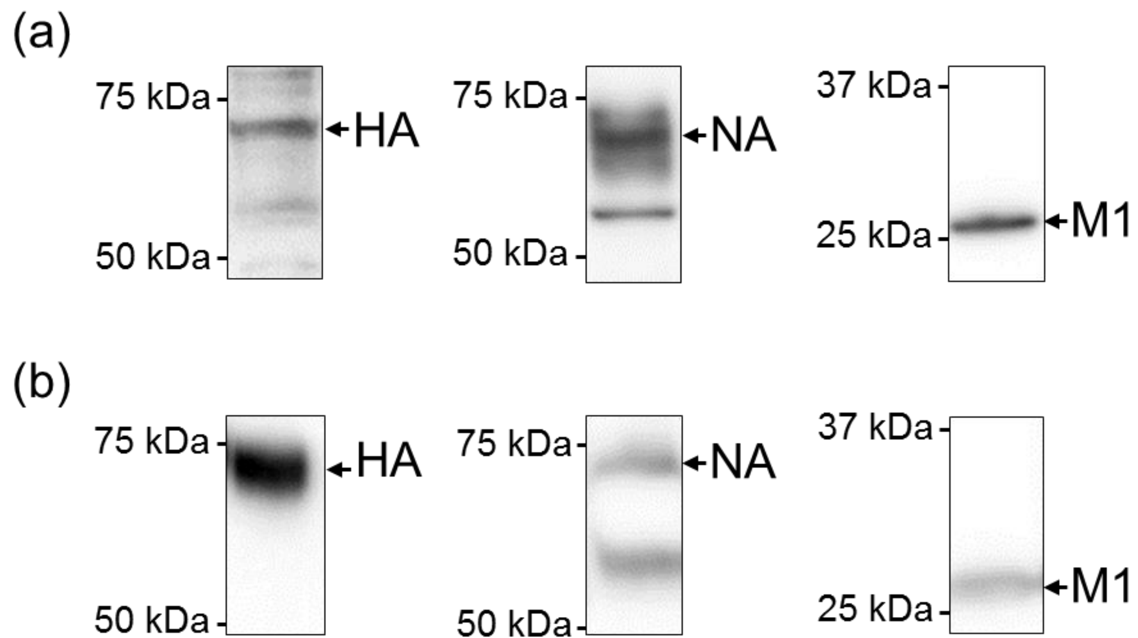


Figure 1. Detection of viral proteins of H17N10 BatIV in the cell lysate and supernatant. HEK293T cells transfected with plasmids were cultured for 48 hours at 37° C. After incubation, cell lysates (a) and supernatants (b) were harvested for western blotting to detect H17 HA, N10 NA, and M1 proteins as described in Materials and Methods.

Morphology of BatIV particles. To observe viral particles in the supernatants of the transfected cells, transmission electron microscopy (TEM) was used for confirming the presence of virions with numerous spikes on their surfaces. Interestingly, there were two morphologically different virus particles with spherical and long filamentous structures (Figure 2). The virion diameter of the spherical particles was approximately 100 nm (the average and standard deviation of 30 randomly selected particles was 105±23 nm, which was similar to that of typical IAV, A/Puerto Rico/8/1934 (H1N1) (96±22 nm). On the other hands, the virion length of the long filamentous particles was over 1000 nm. Densely arrayed spike structures were observed on the surfaces of both viral particles. Immuno-TEM revealed that these spikes were BatIV glycoproteins, H17 HA and N10 NA proteins (Figure 2e-h). No significant difference was found in the overall

morphology between H17N10 and H18N11 BatIV particles.

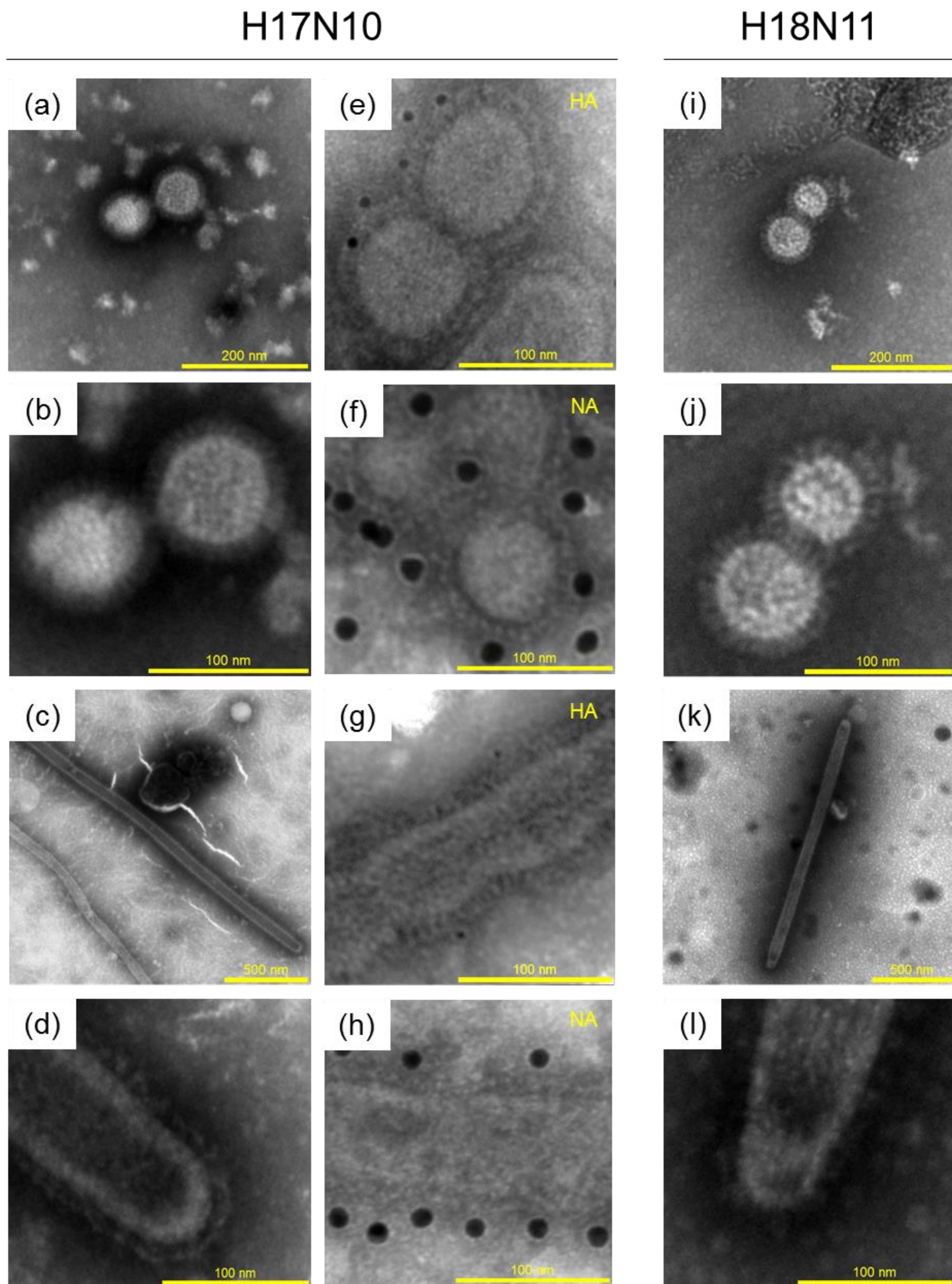


Figure 2. Morphology of BatIV particles. H17N10 (a-h) and H18N11 (i-l) virions in the supernatant were fixed and negatively stained as described in Materials and Methods. For immune transmission electron microscopy, an anti-HA2 monoclonal antibody (e, g) and anti-N10 NA rabbit serum (f, h) were used.

Infection of YubFKT2 cells with BatIV. To confirm the potential of generated BatIV particles to infect cells, first, the cell line YubFKT2, which is derived from the bat (*M. fuliginosus*) was used. BatIV particles were treated with trypsin to cleave the HA protein, which is generally required for activation of IAV HAs, and inoculated into YubFKT2 cells. Forty-eight hours later, the cells were fixed and stained for detecting M1 and HA proteins in immunofluorescent assays (IFA) (Figure 3). Infected cells were clearly stained with the antibodies specific to these viral proteins, suggesting that viral RNA transcription/replication followed by viral protein synthesis occurred in these cells.

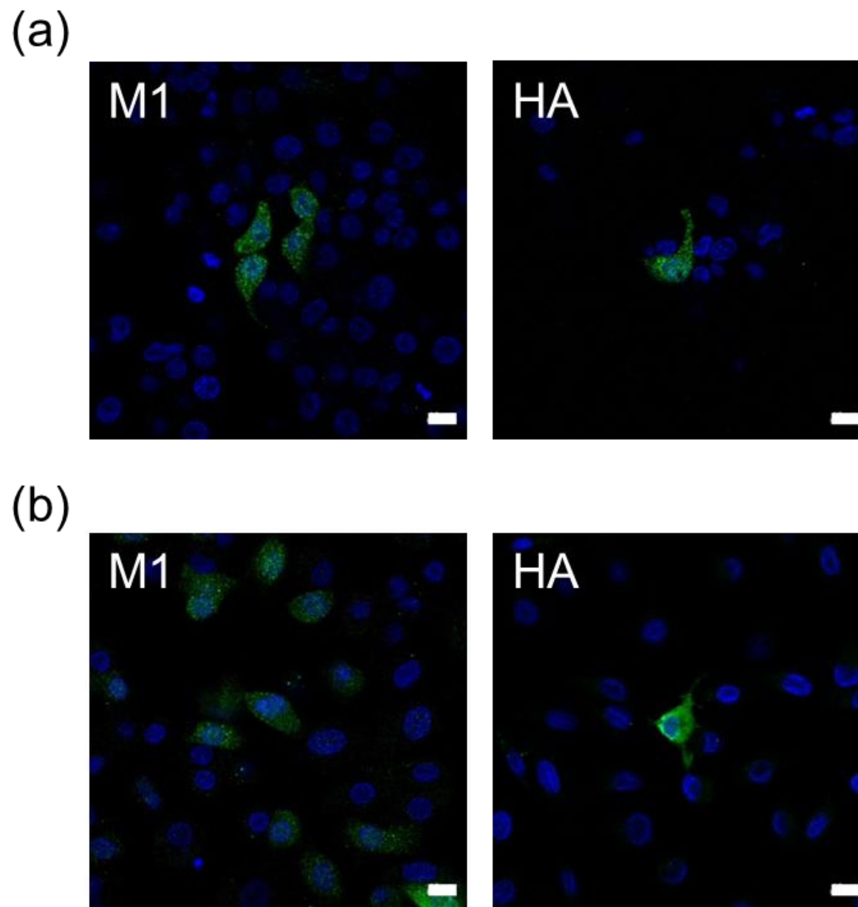


Figure 3. BatIV protein synthesis in bat cells detected by IFA. H17N10 BatIV particles in the supernatant of transfected HEK293T cells were concentrated, treated with trypsin and inoculated into YubFKT2 (a) and SuBK12-08 (b) cells. Forty-eight hours later, cells were fixed and stained for detecting M1 and HA proteins. Scale bars represent 10 µm.

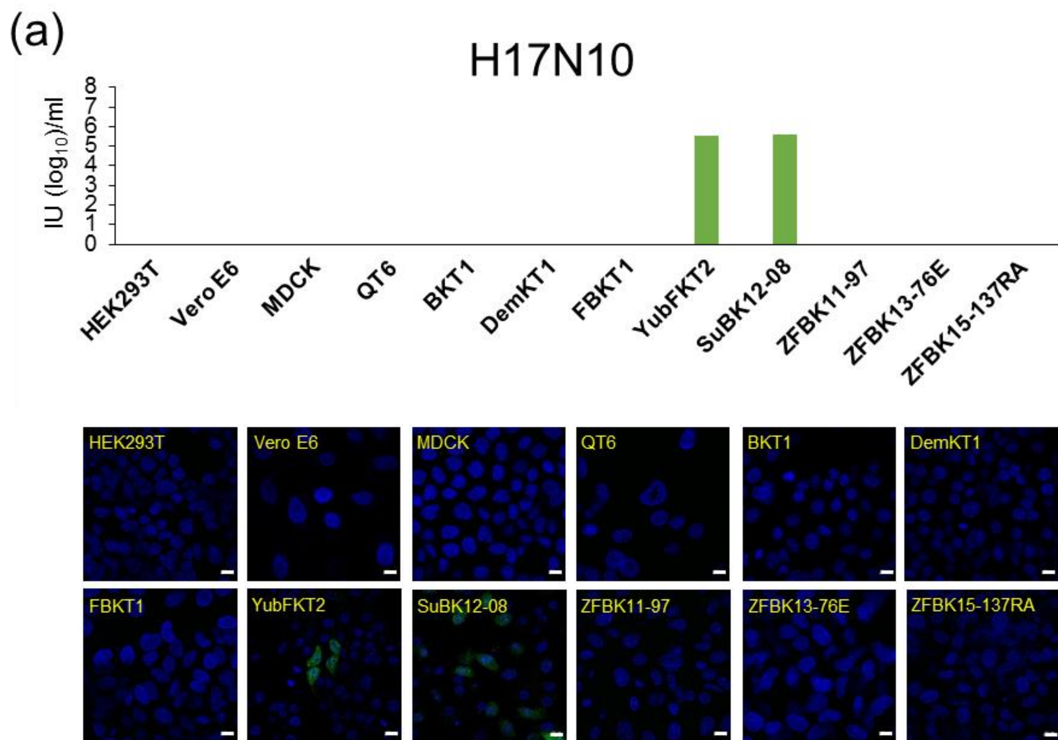
Cell lines susceptible to BatIV. In a previous study, my laboratory screened various cell lines, including bat-derived cells, for their susceptibility to VSV pseudotyped with BatIV glycoproteins and found that the virus infected particular bat cell lines[15]. To confirm this tropism, BatIV particles produced from the transfected HEK293T cells were inoculated into various cultured cells (i.e., 1 avian and 11 mammalian cell lines including 8 that were derived from bats) (Table 1) and the cells were stained for IFA. Among the bat-derived cell lines, the *Miniopterus fuliginosus* and *Miniopterus*

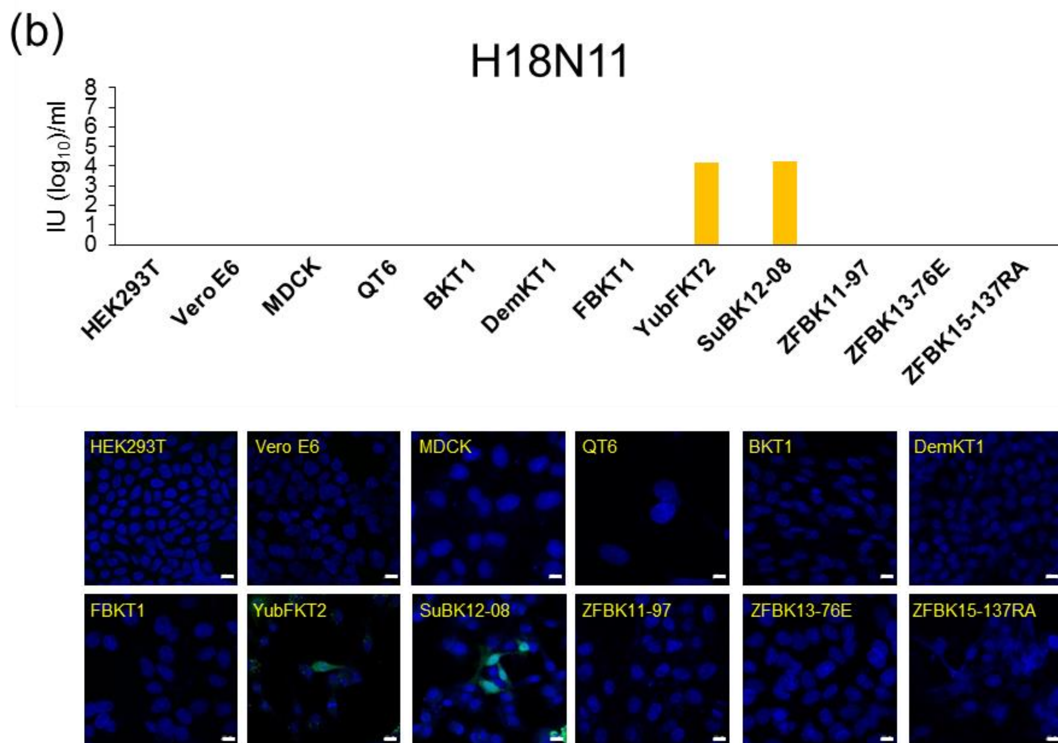
schreibersii bat-derived lines (YubFKT2 and SuBK12-08 cells, respectively) showed susceptibility to BatIV. However, no infected cells were observed in the other mammalian and avian cell lines, including Vero E6, MDCK, and QT6. To quantify the infectivity of BatIV in each cell line, infectious units (IU) were calculated by counting the number of fluorescent cells and found that both H17N10 and H18N11 BatIVs showed similar preference profiles of infectivity (Figure 4a-b). Interestingly, YubFKT2 and SuBK12-08 cells showed susceptibility equivalent to or higher than MDCK II cells which have been reported to be susceptible to BatIVs (Figure 4c)[22].

Table 1. Origins of cellines used in this study

Cell lines	Zoological name	Species
HEK293T	Human	*Homo sapiens*
Vero E6	African green monkey	*Chlorocebus* sp.
MDCK	Dog	*Canis lupus familiaris*
MDCK II	Dog	*Canis lupus familiaris*
QT6	Japanese quail	*Coturnix coturnix japonica*
BKT1	Greater horseshoe bat	*Rhinolophus ferrumequinum*
DemKT1	Leschenault's rousettus	*Rousettus leschenaultii*
FBKT1	Yaeyama flying fox	*Pteropus dasymallus yayeyamae*
YubFKT2	Eastern bent-winged bat	*Miniopterus fuliginosus*
SuBK12-08	Schreiber's bat	*Miniopterus schreibersii*
ZFBK11-97[a)]	Peters's epauletted fruit bat	*Epomophorus crypturus*
ZFBK13-76E	Straw-colored fruit bat	*Eidolon helvum*
ZFBK15-137RA	Egyptian fruit bat	*Rousettus aegyptiacus*

[a)]Temporarily determined by habitat and nucleotide sequence identity of cytochrome *b* genes (97% in basic local alignment search tool (BLAST) search). East African epauletted fruit bat (*Epomophorus minimus*), Ansell's epauletted fruit bat (*Epomophorus anselli*), Peter's dwarf epauletted fruit bat (*Micropteropus pusillus*) and Gambian epauletted fruit bat (*Epomophorus gambianus*) are also genetically similar (97%).


(a)
H17N10
IU (log10)/ml
8
7
6
5
4
3
2
1
0
HEK293T
Vero E6
MDCK
QT6
BKT1
DemKT1
FBKT1
YubFKT2
SuBK12-08
ZFBK11-97
ZFBK13-76E
ZFBK15-137RA


(b)
H18N11
IU (log10)/ml
8
7
6
5
4
3
2
1
0
HEK293T
Vero E6
MDCK
QT6
BKT1
DemKT1
FBKT1
YubFKT2
SuBK12-08
ZFBK11-97
ZFBK13-76E
ZFBK15-137RA

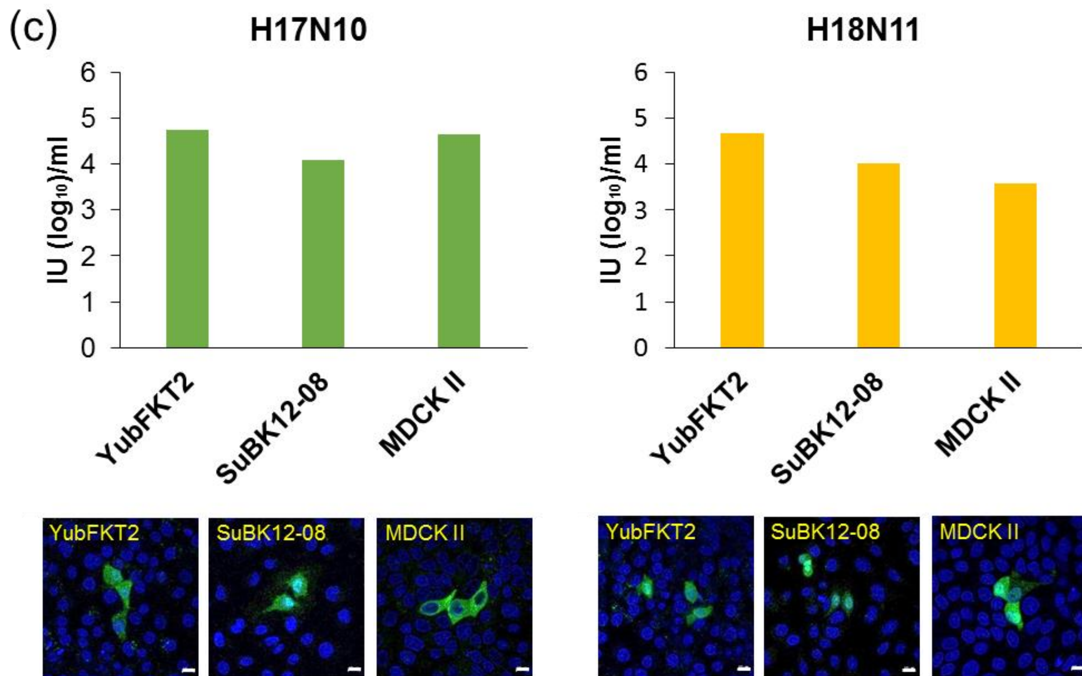


Figure 4. Infectivities of BatIVs in various cell lines. H17N10 (a) and H18N11 (b) BatIVs were inoculated into various cell lines. (c) H17N10 and H18N11 BatIVs were inoculated into YubFKT2, SuBK12-08, and MDCK II cell lines. Infectious units (IUs) of the viruses in different cell lines were determined by counting the number of IFA-positive cells stained with the anti-M1 monoclonal antibody. Scale bars represent 10 μm. Experiments were triplicated and the representative data are shown.

Generation of BatIV reassortants from plasmids. Furthermore, generation of reassortants between the two BatIVs were carried out. HEK293T cells were transfected with plasmids for reassortant viruses whose NA segments were swapped with each other (i.e., H17N11 and H18N10) and those having H17 HA and N10 NA segments with internal protein gene segments of H18N11 BatIV and vice versa. All these reassortant viruses were successfully generated and efficiently infected YubFKT2 cells (Figure 5).

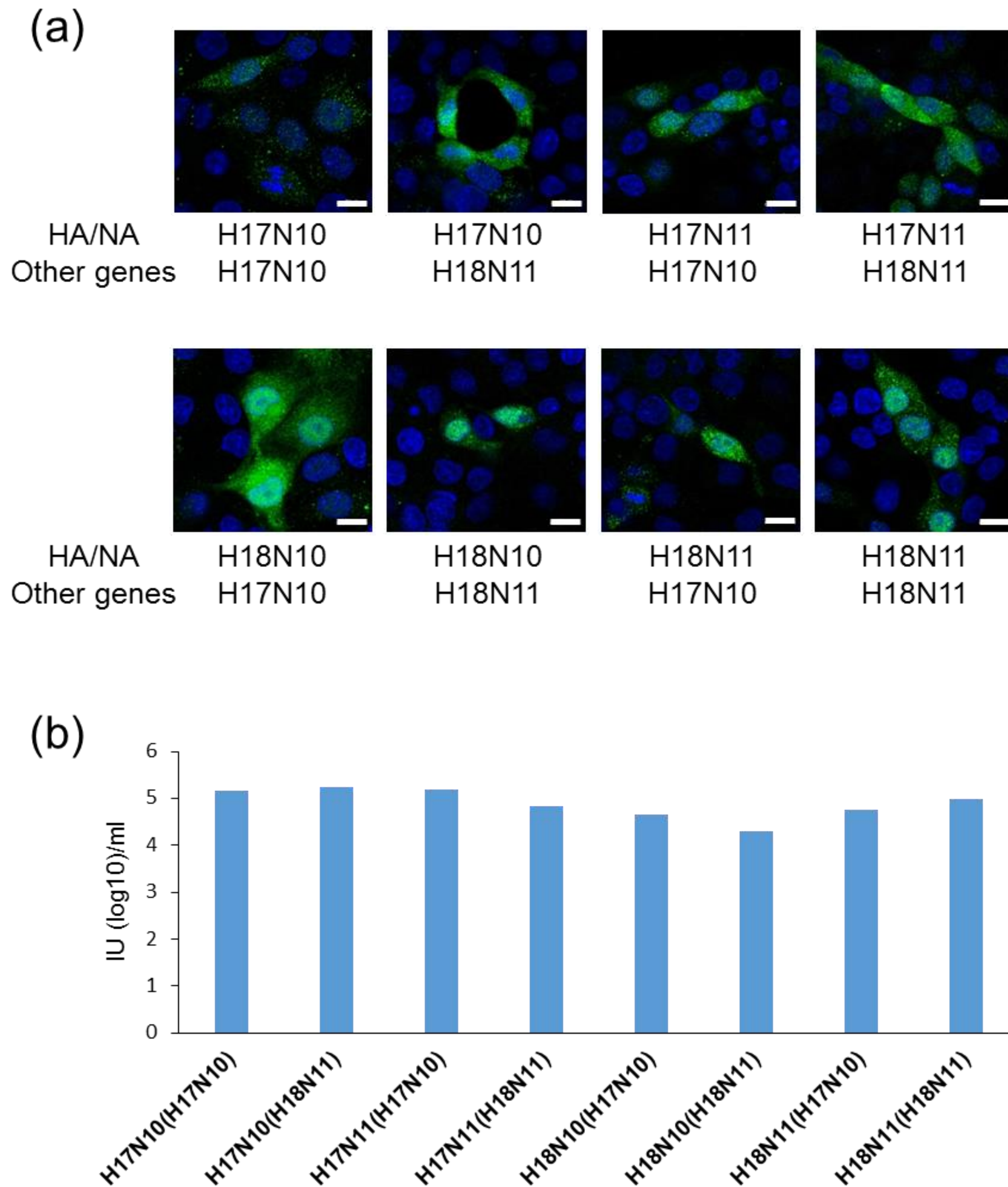


Figure 5. Infectivities of BatIV reassortants. (a) Reassortant viruses consisting of H17 or H18 HA genes, N10 or N11 NA genes, and internal genes of H17N10 or H18N11 were produced with many combinations. Reassortant viruses were inoculated into YubFKT2 cells. Forty-eight hours later, the cells were fixed and stained for the detection of the M1 protein. Scale bars represent 10 μm. (b) Infectious units (IUs) of the viruses were determined by counting the number of IFA-positive cells stained with the anti-M1 monoclonal antibody. Experiments were triplicated and the representative data are shown.

Discussion

Infectious BatIVs have never been isolated from bats[5,6]. Thus, reverse genetics approaches have been used to create recombinant BatIVs. However, some previous studies could only investigate reassortant viruses containing BatIV internal protein gene segments and HA/NA gene segments of well-characterized IAVs and failed to generate wild-type BatIVs, most likely due to the strict host cell specificity (i.e., receptor usage) for propagation of BatIVs[23,24]. Recently, one research group confirmed the rescue of infectious BatIV with its whole gene segments using the cell line MDCK II which is originated from highly passaged MDCK cells and has different properties from the parent cell line commonly used for IAV propagation[17]. In this study, the generation of infectious BatIVs and their reassortants using HEK293T cells were demonstrated.

Consistent with the previous study[22], BatIV proteins were newly synthesized in plasmid-transfected HEK293T cells, indicating that the BatIV ribonucleoprotein complex worked in human kidney cells, leading to the production of progeny virus particles. Interestingly, two morphologically different viral particles were observed in the supernatant of the transfected cells. It is known that virions of IAVs isolated from clinical specimens often have spherical and filamentous shapes[25]. However, it is unclear whether the infectious potential of BatIVs depends on the morphological differences of virions. Further studies are required to clarify the relation between virion morphology and viral infectivity.

Reassortant viruses of BatIVs (e.g., H17N11 and H18N10) were generated. This is the first report of reassortants generated between the two BatIVs that have been discovered so far, indicating that these BatIVs share their packaging signals in their RNA genomes. It is thought that each RNA segment contains packaging signals within

the 3'- and 5'-non-coding and parts of coding regions in the genomic RNA[26]. Previous studies tried to generate reassortant viruses consisting of BatIV segments and those of other previously known IAVs[23,24]. Although BatIV segments have almost identical sequences at the extreme 3'- and 5'-ends, these attempts failed to generate reassortant viruses. However, BatIV-based chimeric viruses whose HA and NA segments have the BatIV non-coding region with adjacent parts of the coding region of well-characterized H1 and H7 HAs were generated in previous studies[23,24]. It was also suggested that the failure of BatIV reassortants with other known IAVs was due to the incompatibility of NP and genomic segments[27]. This evidence implied that the non-coding region and adjacent parts of the coding region might also play critical roles in genome packaging in BatIVs. Taken together, our results suggest the potential for reassortment among BatIVs in nature, though there may be little possibility that BatIVs generate reassortant viruses with avian and mammalian IAVs.

In this study, cells were inoculated with BatIVs from an apical direction. A previous study reported that BatIVs infected preferentially at the basolateral membrane of MDCK II cells[22]. In this study, since BatIV particles were inoculated into sub-confluent cells, it might be possible that infection was initiated at basolateral side, especially the edge of monolayer cells. Alternatively, there may be a difference of expression and localization of BatIV receptor molecule(s) between susceptible bat-derived cell lines and MDCK II cells. It would be of interest to investigate infection through the basolateral site of bat-derived cells. More importantly, BatIV cell tropism is likely determined by the receptor engagement of the virus since the cell lines susceptible to BatIVs were consistent with those of VSVs pseudotyped with BatIV surface glycoproteins[16]. It has been reported that HAs of BatIVs do not recognize sialic

acids, which are known as the receptor molecules of other known IAVs[28]. In addition, crystal structure analysis of BatIV HAs revealed that the receptor binding pocket of BatIV HAs is smaller than those of the other IAVs examined[28,29]. Although the receptor molecules of BatIVs are still unknown, a previous study indicated that BatIV receptor(s) may be a certain glycoprotein shared by some particular bat species[16].

Although the infectiveness of BatIV particles was confirmed by a single-step replication cycle of BatIVs in individual cells, it is unclear that these bat-derived cell lines allow multiple replication cycles of the virus in the presence of trypsin. Like previously known avian and mammalian IAVs, BatIVs also require HA cleavage activation with trypsin-like proteases for their entry into cells[16]. However, since the two BatIV-susceptible cell lines used in this study were highly sensitive to trypsin and also unable to be maintained in fetal calf serum (FCS)-free media, these cell lines could not be used for the cultivation of the virus in the conditions for multiple replication cycles *in vitro*. Our long-term goals are to biologically characterize BatIVs, identify receptor molecules of BatIVs, and elucidate the host range and ecology of BatIVs in nature. Once information on the cellular receptor molecule(s) for BatIVs is in hand, it may be possible to generate fully susceptible cell lines by introducing the molecule(s) and thus to prepare large amounts of infectious BatIV particles needed to fully characterize their biologically properties both *in vitro* and *in vivo*.

Summary

Two novel influenza A virus-like genomes were detected in frugivorous bats in Central and South America. However, the biological properties of these bat-derived influenza viruses (BatIVs) are still largely unknown since infectious viral particles have

never been isolated from the infected host species. In this study, a reverse genetics approach was used to generate infectious BatIV particles entirely from plasmids encoding full-length sequences in eight gene segments. BatIV particles were inoculated into various cell cultures including bat-derived cell lines. BatIVs infected particular bat-derived cells efficiently but not the other cell lines tested. Reassortant viruses between the two BatIVs were also successfully generated and their replication in the susceptible bat cell lines was confirmed. These findings suggest a limited host range and reassortment potential of BatIVs in nature, providing fundamental information for understanding of the ecology of BatIVs.

Chapter II:

Detection of virus-specific antibody in African bats

Introduction

Influenza virus-like RNA genomes (i.e., H17N10 and H18N11) have been discovered in frugivorous bats (*Sturnira lilium* and *Artibeus planirostris*) in Central and South America, respectively[5,6]. However, interestingly, BatIV whose HA is closely related to the H9 subtype were recently isolated from a frugivorous bat species (*Rousettus aegyptiacus*) in Egypt and shown to have several avian IAV characteristics[15].

In Guatemala, 3 of the 316 rectal swab samples of bats were positive for H17N10 BatIV in reverse transcription polymerase chain reaction (RT-PCR) assay[5]. In Peru, 1 of the 110 bat rectal swabs were RT-PCR positive for H18N11 BatIV and serological investigation using blood samples collected from several different bat species indicated a high seroprevalence to BatIV infection[6]. In Ghana, 33 of 100 serum samples were seropositive for one or multiple HA subtypes (e.g. H8, H9, and H12 subtypes)[30]. In Egypt, 105 samples of the 1202 oral and rectal swabs were positive for IAV in an RT-PCR assay[15]. On the other hands, in Central Europe, none of the 1,571 the rectal/oral swab, fecal, and urine samples of bats were RT-PCR-positive for IAV[31].

Although previous studies suggest the worldwide distribution of BatIVs, serological and genetic information on the virus are still limited and the ecology of BatIVs is largely unclear. In this study, to obtain information on the distribution of BatIVs in sub-Saharan Africa, 821 bats of several different species captured in Zambia were screened for the presence of serum antibodies specific to IAVs using HA antigens of H17, H18, and H5 subtypes.

Materials and Methods

Serum samples. Serum samples were collected from wild healthy bats caught by harp traps in Zambia during 2013-2018 (Table 2). All samples were used after heat-inactivation (56°C for 30 minutes).

Construction of plasmids. Nucleotide sequences of HAs of A/little yellow-shouldered bat/Guatemala/060/2010 (H17N10) and A/flat-faced bat/Peru/033/2010 (H18N11) were obtained from Genbank (Accession numbers: CY103892 and CY125945, respectively) and their coding regions were synthesized as described previously[16]. The HA gene of A/duck/Hokkaido/WZ83/2010 (H5N1) (GenBank Accession number: AB612901) was polymerase chain reaction (PCR)-amplified and cloned as described previously[21]. Coding regions of the HA signal peptide and ectodomain of H17, H18, and H5 [1-518 amino acid (aa), 1-515 aa, and 1-519 aa, respectively] were fused with a C-terminal trimerization and a 6×His tag sequences by overlap PCR[32]. The PCR products were cloned into the protein expression vector pCAGGS[20].

HA expression and purification. Expi293F cells (Thermo Fischer Scientific) were transfected with each HA-expressing plasmid using ExpiFectamine 293 reagent (Thermo Fischer Scientific) according to the manufacturer's protocol. Transfected cells were cultured at 37°C in 8% CO_2 with shaking at 0.33 x g for 5 days. The supernatants of the transfected cells were centrifuged at 5000 x g for 20 minutes at 4°C and HA proteins were purified using Ni-NTA agarose (Thermo Fischer Scientific). The purified HA proteins were concentrated and buffer-exchanged into PBS using Amicon ultra

centrifugal filter units (Merck).

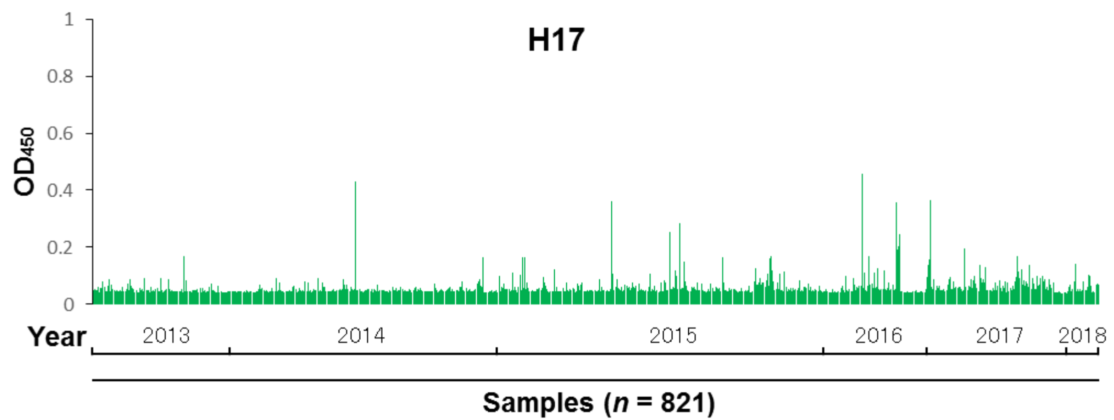
ELISA. Immunoassay plates (Thermo fisher scientific) were coated with each HA protein (2.0 μg / ml) overnight at 4°C. HA-coated plates were blocked with 3% skim milk in PBS at room temperature for two hours. After blocking, the plates were washed with 0.05% Tween 20 in PBS (PBST) once. Serum samples diluted at 1:100 with PBS (50 μl/well) were added into each well and incubated for overnight at 4°C. Then the plates were washed with PBST three times. Second antibodies (1% skim milk PBST with 10,000-fold diluted horseradish peroxidase (HRP)-conjugated Protein A (Calbiochem) and Protein G (ICN biomedicals), respectively) were added into each well and incubated for room temperature, one hour. After incubation, the plates were washed with PBST four times and 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma) was added into each well (50 μl/well). After 20 minutes incubation, 1.0 M phosphoric acid solution was added into each well (50 μl/well) and OD at 450 nm was measured. ELISA was independently conducted twice and average OD values for each sample were used for the analysis.

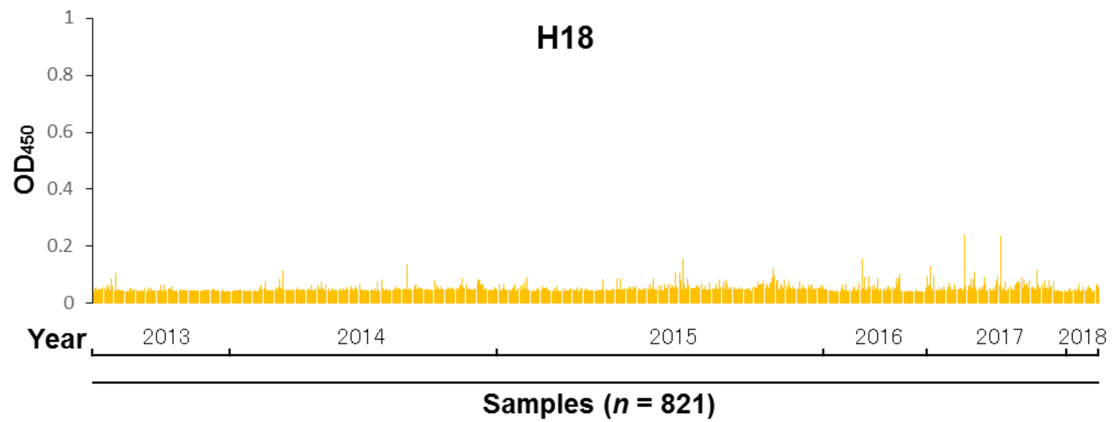
Statistical analysis. In this study, cut-off OD values of each HA antigen were determined as 99% upper confidence bound. To estimate confidence bound, we fitted i) gamma distribution, ii) two-parameter Weibull distribution, iii) three-parameter Weibull distribution by maximum likelihood estimation of model parameters. Three-parameter Weibull distribution was chosen as the best fitted model based on the value of Akaike information criterion. The 99% upper confidence bound of the fitted three-parameter Weibull distribution was calculated as each cut-off value. The cut-off values of each HA

were 0.1562, 0.1086, and 0.2811 for H17, H18, and H5 antigens, respectively.

Results

In this study, 821 serum samples were collected from frugivorous and insectivorous bats in Zambia: 336 *Eidolon helvum*, 296 *Rousettus aegyptiacus*, 69 *Nycteris thebaica*, 60 *Hipposideros gigas*, 36 *Micropteropus pusillus*, 11 *Miniopterus* sp., 6 *Miniopterus schreibersii*, 4 *Myotis* sp., 1 *Rhinolophus* sp., and 2 unidentified bats. These bats were screened for their serum antibodies specific to two BatIVs, A/little yellow-shouldered bat/Guatemala/060/2010 (H17N10) and A/flat-faced bat/Peru/033/2010 (H18N11) and one avian IAV, A/duck/Hokkaido/WZ83/2010 (H5N1) by the HA-based enzyme-linked immunosorbent assay (ELISA) (Figure 6). The optical density (OD) values were analyzed statistically to determine the cutoff OD value for each antigen as described in the Materials and Methods section. Samples that showed OD values above the cutoff value were considered to be positive (Figures 7 and 8). When a sample showed positivity to multiple HA subtypes, the OD values for each HA antigen were compared and the subtype that gave the higher OD value was selected for each positive sample[33]. We found that 20 (2.4%) and 8 samples (1.0%) bound to H17 and H18 HA antigens, respectively, and unexpectedly, 15 samples (1.8%) were regarded as positive for the H5 HA antigen (Tables 2 and 3). These HA-specific antibodies were constantly detected during 2013-2017 (Table 2).





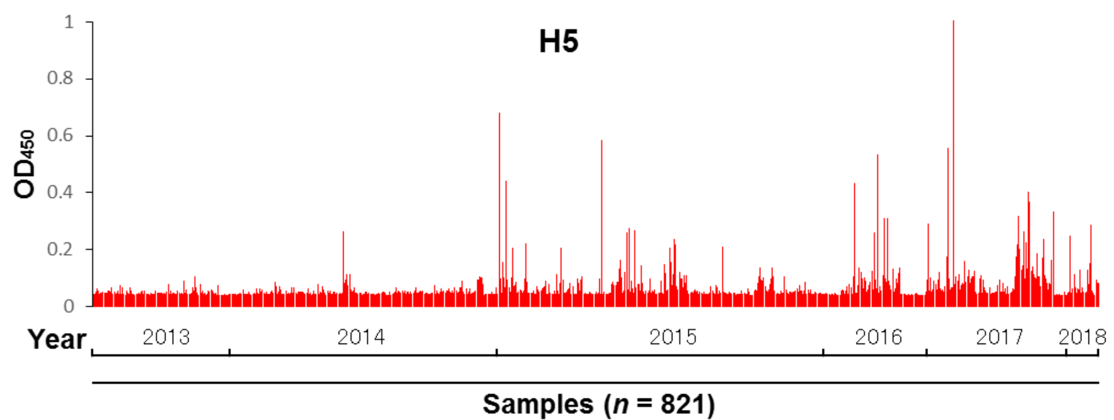
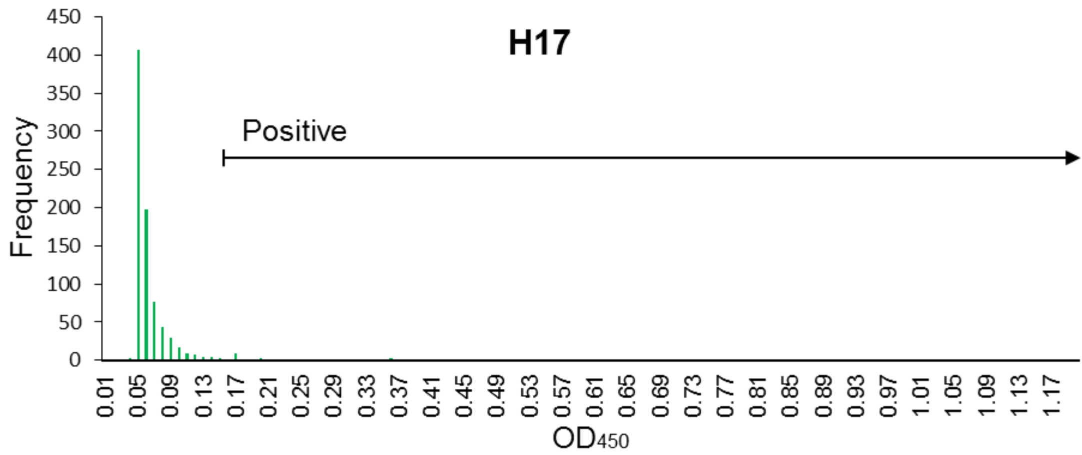
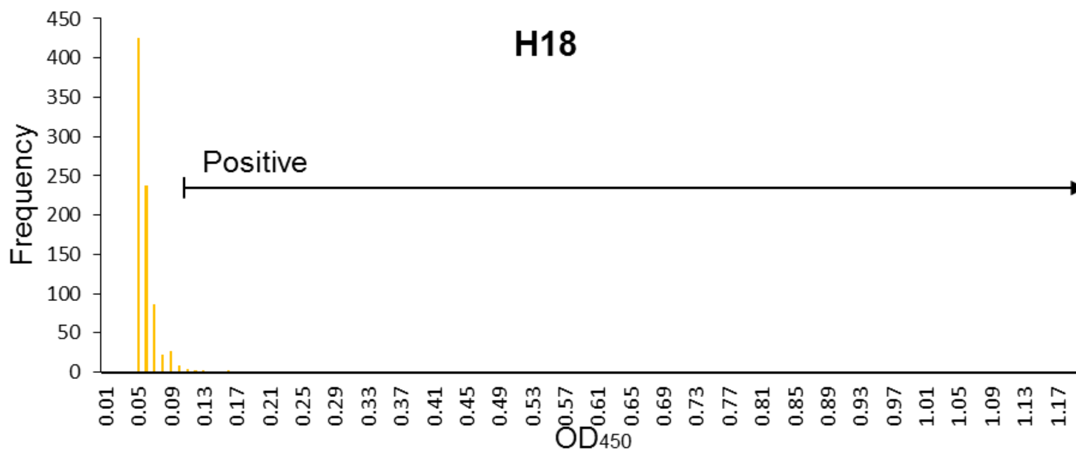


Figure 6. AIV-specific antibodies detected in the sera collected from bats in Zambia. Serum samples were tested (1:100 dilution) for antibodies specific for AIV HAs by ELISA. Optical density values at 450 nm are shown.





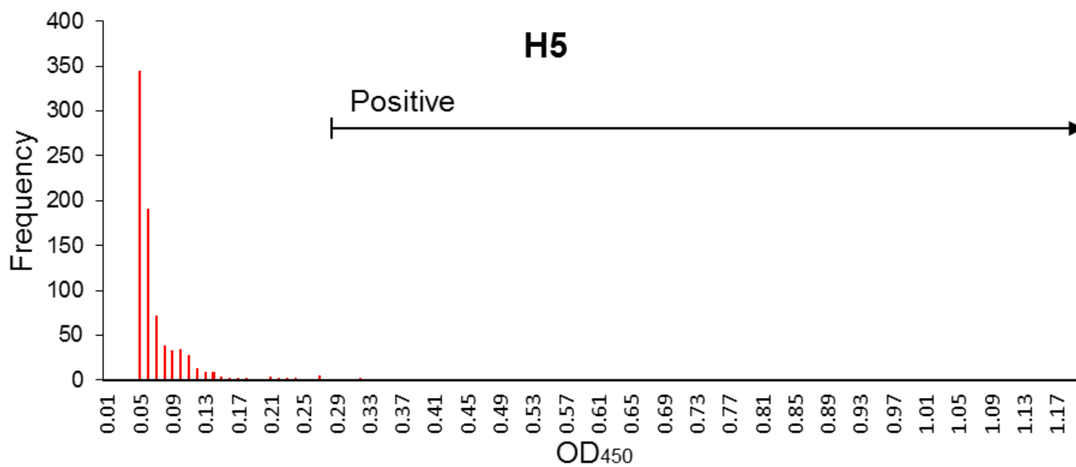
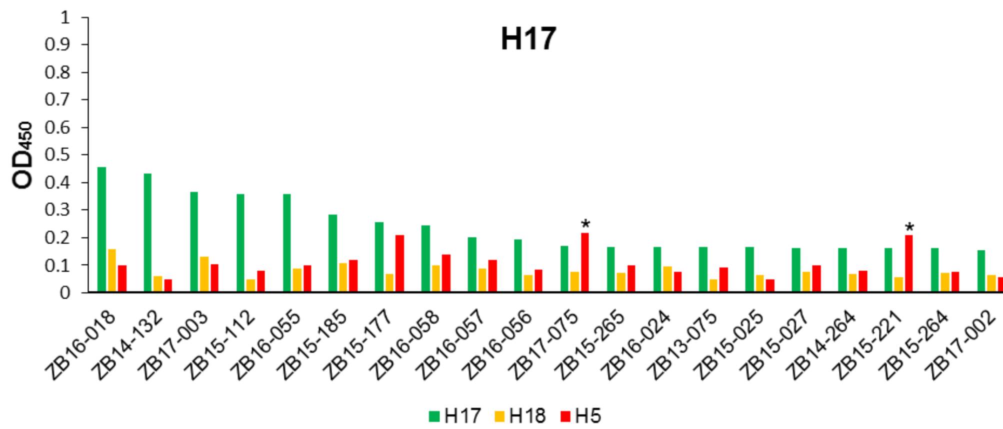
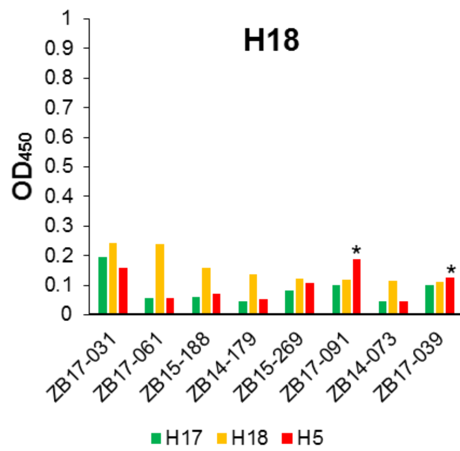


Figure 7. Histograms of all bat serum samples. The histograms represent distribution of OD values to each HA antigen. The OD values larger than 0.1562, 0.1086, and 0.2811 were considered to be positive for H17, H18, and H5, respectively.





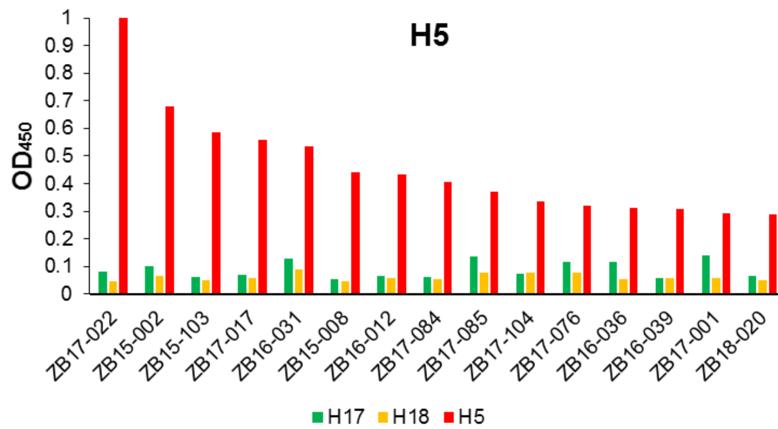


Figure 8. Specificity of serum IgG antibodies detected in fruit bats. Serum samples were tested (1:100 dilution) for antibodies specific for AIV HAs by ELISA. Optical density values at 450 nm are shown. Asterisks represent the values above the statistical cutoff. *Since the cut-off values of H5 HA was 0.2811, these samples were not regarded as positive for H5.

Table 2. Seroprevalence of bats to influenza viruses in each year

Year	Number of samples	H17	H18	H5	Total (Positive)
2013	111	1	0	0	1
2014	220	2	2	0	4
2015	279	8	2	3	13
2016	71	6	0	4	10
2017	114	3	4	7	14
2018	26	0	0	1	1
Total	821	20	8	15	43

Table 3. Seroprevalence to influenza viruses in each bat species

Species	Feeding habit	Number of samples	H17	H18	H5
Eidolon helvum	frugivoire	336	3	3	0
Hipposideros gigas	insectivoire	60	0	0	0
Micropteropus pusillus	frugivoire	36	0	0	0
Miniopterus schreibersii	insectivoire	6	1	0	0
Miniopterus sp.	insectivoire	11	0	0	0
Myotis sp.	insectivoire	4	0	0	0
Nycteris thebaica	insectivoire	69	0	1	0
Rhinolophus sp.	insectivoire	1	0	0	0
Rousettus aegyptiacus	frugivoire	296	16	4	15
Others (unidentified)		2	0	0	0
Total		821	20	8	15

Bat species from which viral specific antibodies were detected are shown in Table 2. Of the 20 H17-positive samples, 16 samples were from *Rousettus aegyptiacus*, while 3 and 1 samples were from *Eidolon helvum* and *Miniopterus schreibersii*, respectively. Of the 8 H18-positive samples, 4, 3, and 1 samples were from *Rousettus aegyptiacus*, *Eidolon helvum*, and *Nycteris thebaica*, respectively. All of the H5-positive

samples were from *Rousettus aegyptiacus*.

OD values to each HA antigens in positive bats are shown in Figure 8. Although most of the H17- or H18-positive samples showed distinct specificity to either of the antigens, three *Rousettus aegyptiacus* serum samples (ZB16-018, ZB17-003, and ZB17-031) were positive for both H17 and H18 HA antigens (As mentioned above, samples with higher OD values were selected in Tables 2 and 3). The H5-positive bat sera showed no appreciable cross reactivity to H17 and H18.

Discussion

In this study, 821 serum samples were screened. The overall seroprevalence of influenza virus infection among the tested bat population was 5.2% (43/821); 20, 8, and 15 positive samples for H17, H18, and H5 subtypes, respectively. Of the bat species tested in this study, *Rousettus aegyptiacus* showed the highest positive rate; 16/296 (5.7%), 4/296 (1.1%), and 15/296 (5.1%) for H17, H18, and H5, respectively. *Eidolon helvum* showed the second highest positive rate; 3/336 (0.9%) and 3/336 (0.9%) for H17 and H18, respectively). These two species were frugivorous bats that are widely distributed in Africa. It is particularly noted that *Rousettus aegyptiacus* bats showed relatively higher positivity and OD values to the H5 antigen. This finding suggests that this bat species might be infected with BatIVs having a novel HA that is antigenically related to avian IAVs of the H5 subtype, which have so far never been detected in any bat species.

It was reported that 30% of the serum samples collected from Ghanan *Eidolon helvum* which is known as a migrating bat species[34] recognized H9 HA antigens derived from avian IAVs[30]. More importantly, a novel H9N2-like BatIV which have avian

IAV-like characteristics was recently isolated from *Rousettus aegyptiacus* in Egypt[15]. Our serological data also suggested that frugivorous bats in Africa may have possibilities to harbor other avian IAV-like BatIVs (e.g., H5 viruses). Although only H17, H18, and H5 HA antigens could be prepared in this study, it is of great interest to investigate serum antibodies of African bats to other HA subtypes.

In the previous study, some cell lines derived from frugivorous bats (*Rousettus aegyptiacus* and *Eidolon helvum*) did not show susceptibility to BatIVs with H17 and H18 HAs most likely due to the lack of the cellular receptors, whereas a cell line derived from *Miniopterus schreibersii*, an insectivorous bat species, was susceptible to these BatIVs[16,35]. However, interestingly, the majority of positive serum samples were from *Rousettus aegyptiacus* and *Eidolon helvum* in this study. Since the cell lines of these frugivorous bats, which are not fully susceptible to BatIVs, are derived from their kidney, it might be possible that H17 and H18 BatIVs infect other organs/tissues that express H17 and H18 HA-specific receptor(s) in these bat species. On the other hands, it might be worthy to note that one (ZB14-264) of the 6 serum samples collected from *Miniopterus schreibersii* was regarded as positive for the H17 HA antigen. This finding suggests that BatIVs may be able to infect insectivorous bats although BatIVs have been thus far detected only in frugivorous bats (i.e. *Sturnira lilium, Artibeus planirostris,* and *Rousettus aegyptiacus*).

In summary, influenza virus-specific antibodies (H17, H18, and H5 subtypes) were detected in sera of bat captured in Zambia. These positive serum samples were mainly from frugivorous bats (i.e. *Rousettus aegyptiacus* and *Eidolon helvum*), suggesting that BatIV infection may be prevalent in African frugivorous bats. More importantly, the presence of anti-H5 antibody in bats may suggest the existence of

BatIVs antigenically closely related to avian influenza viruses with the H5 HA subtype.

Summary

While bat-derived influenza viruses (BatIVs) were detected in frugivorous bats in Central/South America and Egypt, distribution of BatIVs is still largely unknown. In this study, 821 serum samples collected from bats in Zambia were screened for the presence of antibodies specific to H17, H18, and H5 HA antigens. Twenty and 8 serum samples showed reactivities to H17 and H18 HA antigens, respectively, and interestingly, H5 HA-specific antibodies were also detected in 15 samples without any cross reactivity to H17 and H18 HAs. Most of the positive serum samples were from frugivorous bats (i.e. *Rousettus aegyptiacus* and *Eidolon helvum*). These results suggest that African bats may harbor BatIVs including novel viruses that have not been identified previously (e.g., BatIVs having HA closely related to the avian H5 subtype).

Conclusion

Two novel IAV-like genomes (i.e. H17N10 and H18N11) were detected in frugivorous bats in Central and Southern America. However, the biological property and ecological distribution of BatIVs were largely unknown since infectious viral particles of BatIVs have never been isolated from infected animals and information on the prevalence of IAV infection in bats is limited.

In Chapter I, infectious BatIV particles were generated by a reverse genetics approach. It was found that BatIVs infected particular cell lines derived from insectivorous bats (i.e. YubFKT2 and SuBK12-08), confirming their host species specificity. It was also demonstrated that reassortant viruses between BatIVs (e.g., H17N11 and H18N10) were successfully generated, suggesting the potential of reassortment among BatIVs in nature.

In Chapter II, IAV HA-specific antibodies (H17, H18, and H5 subtypes) were detected in the sera of bats captured in Zambia during 2013-2018. The majority of positive serum samples were mainly obtained from frugivorous bats, whereas H17 or H18 HA-specific antibodies were also detected in some of the insectivorous bats. It was particularly of note that anti-H5 antibody was detected in 1.8% of the samples without any cross reactivity to H17 and H18 HAs, suggesting the existence of BatIVs antigenically related to avian H5 influenza viruses in African bats.

The present study provides basic information to understand the biology and ecology of BatIVs and particularly suggests that BatIVs may be capable of infecting both frugivorous and insectivorous bats and distributed widely in nature. However, further studies using infectious BatIVs, as well as increased number of genetic and epidemiological information, are required to fully understand zoonotic potential, host

range, pathogenicity, and evolution of BatIVs.

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Summary in Japanese (和文要旨)

A 型インフルエンザウイルスは、オルソミクソウイルス科に属し、ヒトを含む哺乳類と鳥類に感染する人獣共通感染症病原体である。A 型インフルエンザウイルスは、8 分節のマイナス一本鎖 RNA をゲノムに持ち、表面糖蛋白質であるヘマグルチニン（HA）およびノイラミニダーゼ（NA）の抗原性の違いにより亜型に分類される。16 種類の HA（H1-H16）と 9 種類の NA が自然宿主である野生水禽の中で維持されており、HA と NA の組み合わせによる多様な亜型のウイルスが自然界に存在する。

中南米の食果コウモリから新しい A 型インフルエンザウイルス様ゲノムが検出され、新しいインフルエンザウイルス（H17N10 および H18N11 亜型）として報告されたが、感染性ウイルス粒子が感染動物から分離されていないため、これらのコウモリインフルエンザウイルスの生物学的特性の多くは不明である。第 1 章ではリバースジェネティクス法を用いて H17N10 および H18N11 亜型のコウモリインフルエンザウイルスの感染性粒子を作出した。ウイルスゲノム合成プラスミドとウイルスポリメラーゼ発現プラスミドをトランスフェクションした HEK293T 細胞の細胞内ならびに培養上清中から HA、NA およびウイルスマトリックス蛋白質が検出されたことから、コウモリインフルエンザウイルスのポリメラーゼはヒト由来の細胞株である HEK293T 細胞内で機能することが確認された。培養上清中には球状とフィラメント状の異なる 2 種類の形態を有したウイルス粒子が透過型電子顕微鏡により観察された。作出したウイルス粒子を哺乳類ならびに鳥類由来の細胞株に接種したところ、ユビナガコウモリ属のコウモリ由来の細胞株にのみ感染性を示した。２種のコウモリインフルエンザウイルス（H17N10 および H18N11）の間で HA および NA を入れ換えた遺伝子再集合体ウイルス（H17N11 および H18N10）の作出にも成功し、感受性

を示すコウモリ由来細胞株への感染も確認した。このことから、H17N10 および H18N11 コウモリインフルエンザウイルスは自然界で遺伝子再集合を起こす可能性が示唆された。

H17N10 および H18N11 ウイルスの RNA ゲノムは中南米の食果コウモリから検出されているが、コウモリを対象としたインフルエンザウイルス感染症の疫学調査の報告が限られていることから、その生態学的分布は不明である。第２章では、アフリカ南部のザンビア共和国で捕獲した合計 821 個体の食果および食虫コウモリから採取した血清を用いて、H17、H18 および H5 亜型のインフルエンザウイルスに対する抗体の検出を試みた。精製 HA を抗原とし、酵素標識したプロテイン A および G によって抗体を検出する ELISA 法を用いて 821 検体をスクリーニングした結果、合計 43 検体（5.2%）が陽性となり、亜型ごとでは、H17、H18 および H5 に対してそれぞれ 20 検体（2.4%）、8 検体（1.0%）および 15 検体（1.8%）が陽性であった。検出されたほとんどの抗体は亜型特異的であり、他の亜型との交差反応性を示さなかった。陽性血清の大多数は食果コウモリ（*Rousettus aegyptiacus* および *Eidolon helvum*）由来であったが、食虫コウモリ（*Miniopterus schreibersii*）からも H17 に対する抗体が検出されたことから、食虫コウモリもインフルエンザウイルスに感染することが示唆された。これらの結果は、アフリカのコウモリにおける H17 および H18 亜型のインフルエンザウイルス感染を示唆するとともに、H5 亜型の鳥インフルエンザウイルスに抗原性が類似した HA を持つコウモリインフルエンザウイルスの存在を示唆している。

本研究では、遺伝子配列のみが知られていたコウモリインフルエンザウイルスの感染性粒子および遺伝子再集合体の作出に成功し、感受性を示す細胞株を特定した。また、ザンビア共和国で捕獲したコウモリの血清中にインフル

エンザウイルスの HA に反応性を示す抗体が検出されたことから、アフリカ南部に生息するコウモリにインフルエンザウイルスが感染している可能性が示唆された。以上のことから、本研究はコウモリインフルエンザウイルスの生物学的特性と生態学的分布を理解するための重要な情報を提供した。しかし、本ウイルスの病原性、人獣共通感染症病原体としての可能性、宿主域、分布域および進化過程に関する情報は依然として限られるため、今後の更なる研究が必要である。

References

1. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**, 152-179 (1992)
2. Röhm, C., Zhou, N., Süss, J., Mackenzie, J. & Webster, R. G. Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. *Virology.* **217**, 508-516 (1996)
3. Fouchier, R. A., Munster, V., Wallensten, A., Bestebroer, T. M., Herfst S., Smith D., Rimmelzwaan G. F., Olsen B. & Osterhaus A. D. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79**, 2814-2822 (2005)
4. A revision of the system of nomenclature for influenza viruses: a WHO memorandum. *Bull. WHO* **58**, 585-591 (1980)
5. Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D.A., Chen, L. M., Recuenco, S., Ellison, J. A., Davis, C. T., York, I. A., Turmelle, A. S., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M. R., Tang, K., Rowe, L. A., Sammons, S., Xu, X., Frace, M., Lindblade, K. A., Cox, N. J., Anderson, L. J., Rupprecht, C. E. & Donis, R. O. A distinct lineage of influenza A virus from bats. *Proc. Natl. Acad. Sci. U. S. A*. **109**, 4269-4274 (2012)
6. Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J., Chen, L. M., Johnson, A., Tao, Y., Dreyfus, C., Yu, W., McBride, R., Carney, P. J., Gilbert, A. T., Chang, J., Guo, Z., Davis, C. T., Paulson, J. C., Stevens, J., Rupprecht, C. E., Holmes, E. C., Wilson, I. A. & Donis, R. O. New world bats harbor diverse influenza A viruses. *PLoS Pathog*.

9, e1003657 (2013)

7. Zhu, X., Yu, W., McBride, R., Li, Y., Chen, L. M., Donis, R. O., Tong, S., Paulson, J. C. & Wilson, I. A. Hemagglutinin homologue from H17N10 bat influenza virus exhibits divergent receptor-binding and pH-dependent fusion activities. *Proc. Natl. Acad. Sci. U. S. A*. **110**, 1458-1463 (2013)
8. García-Sastre, A. The neuraminidase of bat influenza viruses is not a neuraminidase. *Proc. Natl. Acad. Sci. U. S. A*. **109**, 18635-18636 (2012)
9. Ma, W., García-Sastre, A. & Schwemmle, M. Expected and unexpected features of the newly discovered bat influenza A-like viruses. *PLoS Pathog*. **11**, e1004819 (2015)
10. Jones, K. E., Purvis, A., MacLarnon, A., Bininda-Emonds, O. R. & Simmons, N. B. A phylogenetic supertree of the bats (Mammalia: Chiroptera). *Biol. Rev. Camb. Philos. Soc.* **77**, 223–259 (2002)
11. Towner, J. S., Amman, B. R., Sealy, T. K., Carroll, S. A., Comer, J. A., Kemp, A., Swanepoel, R., Paddock, C. D., Balinandi, S., Khristova, M. L., Formenty, P. B., Albarino, C. G., Miller, D. M., Reed, Z. D., Kayiwa, J. T., Mills, J. N., Cannon, D. L., Greer, P. W., Byaruhanga, E., Farnon, E. C., Atimnedi, P., Okware, S., Katongole-Mbidde, E., Downing, R., Tappero, J. W., Zaki, S. R., Ksiazek, T. G., Nichol, S. T. & Rollin, P. E. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog*. **5**, e1000536 (2009)
12. Plowright, R. K., Eby, P., Hudson, P. J., Smith, I. L., Westcott, D., Bryden, W. L., Middleton, D., Reid, P. A., McFarlane, R. A., Martin, G., Tabor, G. M., Skerratt, L. F., Anderson, D. L., Crameri, G., Quammen, D., Jordan, D., Freeman, P., Wang, L. F., Epstein, J. H., Marsh, G. A., Kung, N. Y. &

McCallum, H. Ecological dynamics of emerging bat virus spillover. *Proc. Biol. Sci*. **282**, 20142124 (2015)

13. Smith, I. & Wang, L. F. Bats and their virome: An important source of emergingviruses capable of infecting humans. *Curr Opin Virol.* **3**, 84–91 (2013)

14. Memish, Z. A., Mishra, N., Olival, K. J., Fagbo, S. F., Kapoor, V., Epstein, J. H., AlHakeem, R, Durosinloun, A., Al Asmari, M., Islam, A., Kapoor, A., Briese, T., Daszak, P., Al Rabeeah, A. A. & Lipkin, W. I. Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. *Emerg. Infect. Dis*. **19**, 1819–1823 (2013)

15. Kandeil, A., Gomaa, M. R., Shehata, M. M., El Taweel, A. N., Mahmoud, S. H., Bagato, O., Moatasim, Y., Kutkat, O., Kayed, A. S., Dawson, P., Qiu, X., Bahl, J., Webby, R. J., Karesh, W. B., Kayali, G., Ali, MA. Isolation and characterization of 1 a distinct influenza A virus from Egyptian bats. *J Virol*. pii: JVI.01059-18 (2018)

16 Maruyama, J., Nao, N., Miyamoto, H., Maeda, K., Ogawa, H., Yoshida, R., Igarashi, M. & Takada, A. Characterization of the glycoproteins of bat-derived influenza viruses. *Virology*. **488**, 43-50 (2015)

17. Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann, E., Hobom, G. & Kawaoka, Y. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. U. S. A*. **96**, 9345-9350 (1999)

18. Maeda, K., Hondo, E., Terakawa, J., Kiso, Y., Nakaichi, N., Endoh, D., Sakai, K., Morikawa, S. & Mizutani, T. Isolation of novel adenovirus from fruit bat

(*Pteropus dasymallus yayeyamae*). *Emerg. Infect. Dis*. **2**, 347-349 (2008)

19. Maruyama, J., Miyamoto, H., Kajihara, M., Ogawa, H., Maeda, K., Sakoda, Y., Yoshida, R. & Takada, A. Characterization of the envelope glycoprotein of a novel filovirus, Lloviu virus. *J. Virol.* **88**, 99-109 (2014)
20. Niwa, H., Yamamura, K. & Miyazaki, J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*. **108**, 193-199 (1991)
21. Nao, N., Kajihara, M., Manzoor, R., Maruyama, J., Yoshida, R., Muramatsu, M., Miyamoto, H., Igarashi, M., Eguchi, N., Sato, M., Kondoh, T., Okamatsu, M., Sakoda, Y., Kida, H. & Takad,a A. A single amino acid in the M1 protein responsible for the different pathogenic potentials of H5N1 highly pathogenic avian influenza virus strains. *PLoS ONE*. **10**, e0137989 (2015)
22. Moreira, É. A., Locher, S., Kolesnikova, L., Bolte, H., Aydillo, T., García-Sastre, A., Schwemmle, M. & Zimmer, G. Synthetically derived bat influenza A-like viruses reveal a cell type- but not species-specific tropism. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 12797-12802 (2016)
23. Juozapaitis, M., Aguiar, Moreira, É., Mena, I., Giese, S., Riegger, D., Pohlmann, A., Höper, D., Zimmer, G., Beer, M., García-Sastre, A. & Schwemmle, M. An infectious bat-derived chimeric influenza virus harbouring the entry machinery of an influenza A virus. *Nat. Commun*. **5**, 4448 (2014)
24. Zhou, B., Ma, J., Liu, Q., Bawa, B., Wang, W., Shabman, R. S., Duff, M., Lee, J., Lang, Y., Cao, N., Nagy, A., Lin, X., Stockwell, T. B., Richt, J. A., Wentworth, D. E. & Ma, W. Characterization of uncultivable bat influenza virus using a replicative synthetic virus. *PLoS Pathog*. **10**, e1004420 (2014)

25. Rossman, J. S. & Lamb, R. A. Influenza virus assembly and budding. *Virology*. **411**, 229-236 (2011)

26. Fujii, Y., Goto, H., Watanabe, T., Yoshida, T., & Kawaoka, Y. Selective incorporation of influenza virus RNA segments into virions. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 2002-2007 (2003)

27. Moreira, É. A., Weber, A., Bolte, H., Kolesnikova, L., Giese, S., Lakdawala, S., Beer, M., Zimmer, G., García-Sastre, A., Schwemmle, M. & Juozapaitis, M. A conserved influenza A virus nucleoprotein code controls specific viral genome packaging. *Nat. Commun.* **7**, 12861 (2016)

28. Sun, X., Shi, Y., Lu, X., He, J., Gao, F., Yan, J., Qi, J. & Gao, G. F. Bat-derived influenza hemagglutinin H17 does not bind canonical avian or human receptors and most likely uses a unique entry mechanism. *Cell Rep*. **3**, 769-778 (2013)

29. Wu, Y., Wu, Y., Tefsen, B., Shi, Y. & Gao, G. F. Bat-derived influenza-like viruses H17N10 and H18N11. *Trends Microbiol.* **4**, 183-191 (2014)

30. Freidl, G. S., Binger, T., Müller, M. A., de Bruin, E., van Beek, J., Corman, V. M., Rasche, A., Drexler, J. F., Sylverken, A., Oppong, S. K., Adu-Sarkodie, Y., Tschapka, M., Cottontail, V. M., Drosten. C. & Koopmans, M. Serological evidence of influenza A viruses in frugivorous bats from Africa. *PLoS One*. 10(5):e0127035 (2015)

31. Fereidouni, S., Kwasnitschka, L., Balkema, Buschmann, A., Müller, T., Freuling, C., Schatz, J., Pikula, J., Bandouchova, H., Hoffmann, R., Ohlendorf, B., Kerth, G., Tong, S., Donis, R., Beer, M. & Harder, T., No virological evidence for an influenza A - like virus in European bats. *Zoonoses Public Health*. 62(3), 187-9 (2015)

32. Lee, P. S., Yoshida, R., Ekiert, D. C., Sakai, N., Suzuki, Y., Takada, A., Wilson, I. A. Heterosubtypic antibody recognition of the influenza virus hemagglutinin receptor binding site enhanced by avidity. *Proc Natl Acad Sci U. S. A.* 109(42):17040-5 (2012)

33. Changula, K, Kajihara, M, Mori-Kajihara, A, Eto, Y, Miyamoto, H, Yoshida, R, Shigeno, A, Hang'ombe, B, Qiu, Y, Mwizabi, D, Squarre, D, Ndebe, J, Ogawa, H, Harima, H, Simulundu, E, Moonga, L, Kapila, P, Furuyama, W, Kondoh, T, Sato, M, Takadate, Y, Kaneko, C, Nakao, R, Mukonka, V, Mweene, A & Takada, A. Seroprevalence of Filovirus Infection of Rousettus aegyptiacus Bats in Zambia. *J Infect Dis*. 218(suppl_5):S312-S317 (2018)

34 Richter, H. V. & Cumming, G. S. First application of satellite telemetry to track African straw‐coloured fruit bat migration. *J. Zool.* 275, 2, 172-176 (2008)

35. Sato, M., Maruyama, J., Kondoh, T., Nao, N., Miyamoto, H., Takadate, Y., Furuyama, W., Kajihara, M., Ogawa, H., Manzoor, R., Yoshida, R., Igarashi, M. & Takada, A. Generation of bat-derived influenza viruses and their reassortants. *Sci Rep*. In press