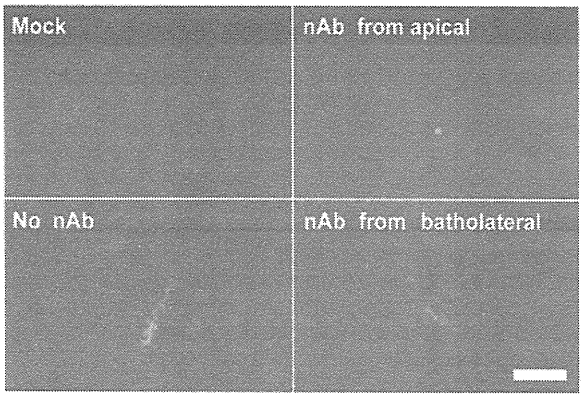

Figure 5. The cell-to-cell transmission of the NA-deficient influenza virus occurs the apical cell surface. Confluent MDCK cells were prepared in transwell inserts and infected with the NA-deficient influenza virus at MOI of 0.0001 in the presence or absence of 0.3% (v/v) antiserum containing neutralizing antibodies (nAb) to influenza A virus. After virus adsorption, the antiserum was added from apical or basolateral side. GFP fluorescence derived from the recombinant virus was observed at 36 hpi. The antiserum added from the apical side could markedly block the cell-to-cell transmission of the NA-deficient influenza virus, whereas the antiserum added from the basolateral side could not. Scale bar, 100 μm.
doi:10.1371/journal.pone.0028178.g005

that progeny virus particles remain on the surface of infected cell even after budding, and can infect the cell previously infected, as well as uninfected cells adjacent to the infected cell, when oseltamivir is present.

Discussion

With the except for the virus which spreads through the cell-cell fusion transmission, virus infection is initiated by the binding of *cell-free* virions to their host cells. Recently, the virus transmission mechanism from an infected cell to adjacent cells without virus diffusion into the extracellular environment is highlighted from the aspect of its significance in virus spreading in the presence of antibodies [1,2]. This antibody-insensitive pathway is often called cell-to-cell transmission [2]. The cell-to-cell transmission may be categorized into two pathways, *i.e.*, transmission of *cell-free* virions to adjacent uninfected cells, and transmission of progeny virions associated on the surface of an infected cell even after budding through narrow synaptic space between an infected cell and adjacent uninfected cells. As an example of the former mechanism, *cell-free* vaccinia virus particles associated with the filopodium of an infected cell are repelled toward neighboring uninfected cells by inducing the formation of actin filament [3]. Several cases have been reported for the latter mechanism: Immunotropic viruses including retroviruses utilize the immunological synapses [4–7]. Immune cells are not constitutively polarized, but contain the machinery that directs their secretory apparatus towards a cell that is involved in an immunological synapse. This machinery can be subverted by retroviruses containing human immunodeficiency virus (HIV). An HIV-infected cell can polarize viral budding towards a target cell expressing receptor through a structure called a virological synapse. Virions bud from an infected cell into a synaptic cleft, from which they fuse with the target-cell plasma membrane [49–52]. The progeny virions of HCV are trapped between infected and uninfected cell membranes at the tight junction. Using Claudin-1 known as a component of the tight junction and one of the entry factors of HCV [8], virions fuse with and penetrate uninfected target cells [31]. Therefore, HCV may acquire the ability to spread within polarized liver epithelium. Thus, the cell-to-cell transmission certainly plays significant roles for the dissemination of several enveloped viruses. However, the cell-to-cell transmission of influenza virus has not been discussed well. Here, we have shown that influenza virus spreads by forming infected cell clusters even in the presence of an NA inhibitor. Live cell imaging clearly showed that influenza virus lacking the NA activity spreads from an infected cell to adjacent cells through the cell-to-cell transmission mechanism (Figure 2). This was also the case for wild-type influenza virus during early phases of infection (Figure 4B). In the cell-to-cell transmission of influenza virus, progeny virions could remain associated with the surface of infected cell even after budding, and then these progeny virions can be passed on to adjacent uninfected cells.

We showed that the cell-to-cell transmission of the NA-deficient influenza virus depends on functional HA. The viral spreading was dramatically suppressed without HA activation by trypsin treatment (Figure 4A). Moreover, the cell-to-cell transmission was also blocked by amantadine, which inhibits the acidification of endosomes required for uncoating of influenza virus particles in endosomes [33,34]. These findings indicate that functional HA and endosome acidification by M2 ion channel are required for the cell-to-cell influenza virus transmission, thereby allowing viruses to enter the adjacent cells through the endocytotic pathway (Figure 4).

Our findings showed that the NA-deficient influenza virus is not diffused into the extracellular environment. The viral spreading in the absence of oseltamivir appears to be much faster compared to the viral spreading in the presence of the drug, suggesting that NA could be involved in determination of spreading speed (Figure 4B). The NA activity prevented progeny virions from entering cells which virus came from (Figure 6), implying that progeny virus particles should be transmitted to adjacent uninfected cells. The cell-to-cell transmission started in early phase of infection, and the virus spread through diffusion of *cell-free* viruses (Figure 4B). Indeed, it was reported that the cell-to-cell transmission is a rapid spreading pathway in the case of vaccinia virus [3]. Vaccinia virus induces a blocking mechanism of superinfection and thereby infects to adjacent uninfected cells efficiently. In early phases of vaccinia virus infection, viral proteins A33 and A36 are expressed at the infected cell surface. Once *cell-free* virus particles contact the filopodium, the A33/A36 complex induces the formation of actin filament, which causes this superinfected virion to be repelled toward uninfected cells [3]. Influenza viruses can re-infect the cells previously infected in the presence of oseltamivir (Figure 6), suggesting that a progeny virion may be bridged by HA between infected and adjacent uninfected cells temporarily. Thus, in the case of the cell-to-cell transmission of influenza virus, we propose that progeny virions associated with the surface of infected cells even after budding are directed to adjacent uninfected cells. The cell-to-cell transmission mechanism of influenza virus is distinctly different from that of vaccinia virus in the infecting virus status: Infected cell-associated virions and *cell-free* virions are involved in the cell-to-cell transmission of influenza virus and vaccinia virus, respectively. The strategy for influenza virus appears to be similar to that for HCV. HCV progeny virions budded from an infected cell are trapped between infected and uninfected adjacent cell membranes at the tight junction. HCV virions then, enter into adjacent cells through endocytosis and low pH-dependent membrane fusion using Claudin-1 [8]. The cell-to-cell transmission of influenza virus also required functional HA and endosome acidification by M2 ion channel. However, it has not been reported that HCV has a gene encoding a receptor destroying

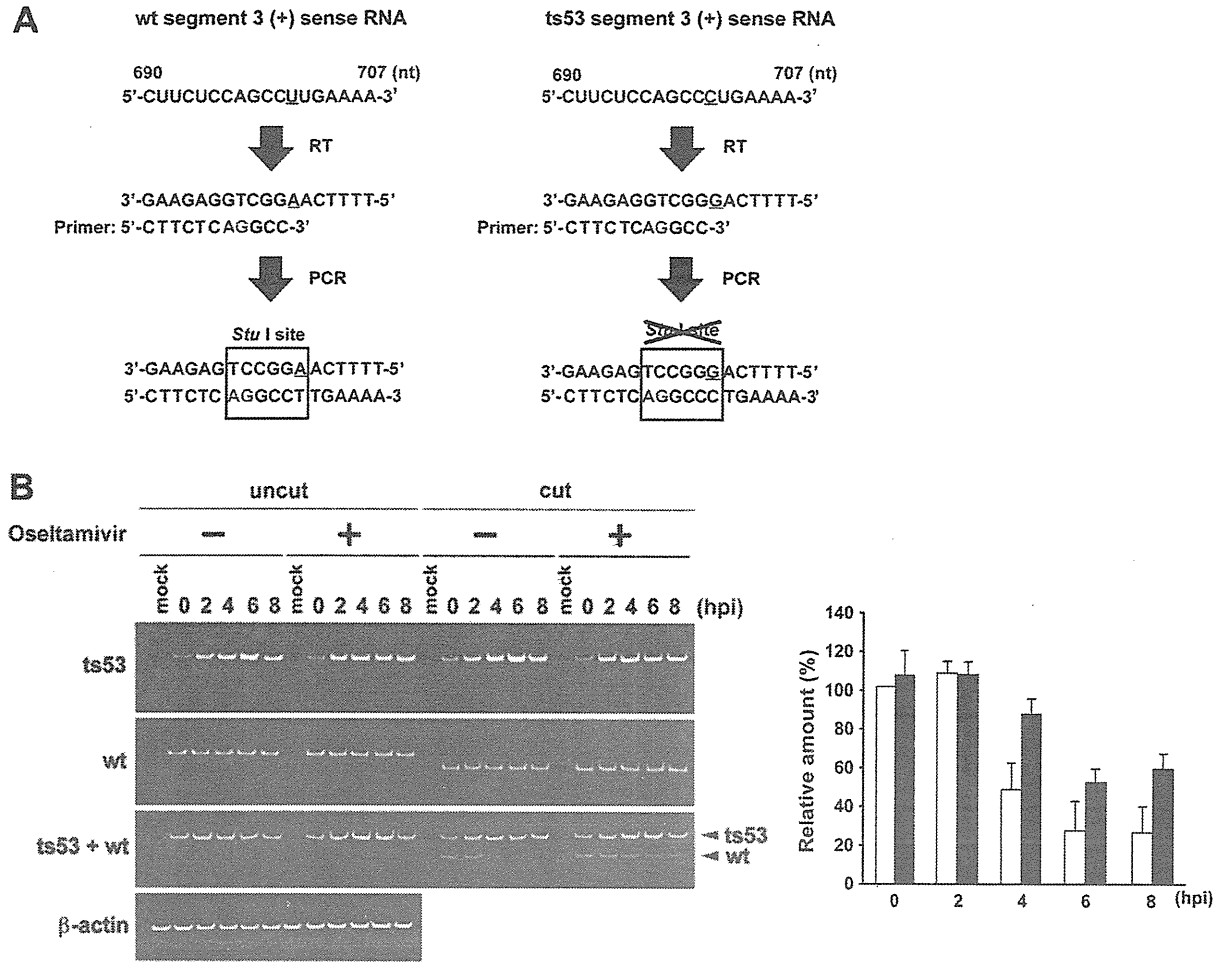

Figure 6. Influenza viruses can not re-infect previously infected cells. (A) A method for determination of the amount of segment 3 genome derived from *ts*53 and wild-type. Total RNA was reverse-transcribed with the primer PA-895-rev, which is complementary to the segment 3 positive-sense RNA. The cDNA was amplified by PCR using primers, PA-895-rev and PA-695-cut partially corresponding to segment 3 positive sense RNA between the nucleotide sequence positions 678 to 700 except for 696 and 697, which are shown in red letters. Since segment 3 of *ts*53 has a substitution mutation from U to C at the nucleotide position of 701, the PCR product derived from wild-type could be digested by *Stu* I but not that from *ts*53. Then, PCR products were digested with *Stu* I and separated through 8% PAGE. (B) Detection of the genome of the segment 3 derived from *ts*53 or wild-type. At 3 hours post superinfection of wild-type virus, total RNA was extracted, and semi-quantitative RT-PCR was performed. Subsequently, the amplified DNA products were digested with *Stu* I and separated through 8% PAGE. Large and small fragments derived from *ts*53 and wild-type viruses were 220 and 199 base pairs, respectively. The relative amount of wild-type segment 3 to that at 0 hour in the absence of oseltamivir phosphate was shown in the graph. Error bars indicate S.D. from 3 independent experiments. White bar, in the absence of oseltamivir phosphate; black bar, in the presence of oseltamivir phosphate.
doi:10.1371/journal.pone.0028178.g006

enzyme similar to NA of influenza virus. We speculated that HCV progeny particles are bridged between infected and adjacent uninfected cells temporarily like influenza virus in the presence of oseltamivir. Progeny influenza virus particles could be transmitted to adjacent uninfected cells efficiently in the presence of the NA activity, suggesting that the cell-to-cell transmission of influenza virus is more strategic than that of HCV.

Our findings raise an interesting question as to what is the biological significance of cell-to-cell transmission for influenza virus infection *in vivo*. Until now, it had been believed that influenza virus was released from infected cells as *cell-free* virions and then spread from cell to cell as well as from organism to organism. The transmission mode by *cell-free* virions undergoes the extremely high-speed of its diffusion and causes epidemic or pandemic infection. The tropism in an infected animal body is generally restricted to respiratory tract or lung and its periphery, and the requirement of a trypsin-like protease has been generally described for the reason of the restriction. It is possible that the cell-to-cell transmission mode may play a significant role for the virus spreading inside of organism, although *cell-free* influenza virions are causative of high-speed spreading. At the least, the limited but distinct level of infection followed by replication could provide some opportunity to generate influenza virus variants. It is an open question whether the cell-to-cell transmission mode is involved in the pathogenesis caused by influenza virus infection *in vivo*.

The existence of cell-to-cell transmission pathway gives a caution when NA inhibitors are used, because NA inhibitors may not be sufficient to completely block the spread of influenza

virus in local microenvironments. Since this cell-to-cell transmission pathway exists, development of antiviral therapeutic strategies in addition to NA inhibitors is highly recommended.

Materials and Methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were kindly gifted by A. Ishihama (Hosei University), and maintained in minimal essential medium (MEM) (Nissui) containing 10% fetal bovine serum. Human embryonic kidney 293T cells were kindly gifted by Y. Kawaoka (University of Tokyo), and maintained in Dulbecco modified Eagle medium (DMEM) (Nissui) supplemented with 10% fetal bovine serum. Influenza virus A/Udorn/72 was grown in allantoic sacs of 11 day-old embryonated eggs (MIYAKE HATCHERY). Wild-type influenza virus A/WSN/33 and *ts*53 mutant were used after single-plaque isolation. MDCK cells were infected with influenza virus A/WSN/33 or *ts*53 at a multiplicity of infection (MOI) of 0.1 PFU/cell, and incubated at 37°C and 34°C, respectively. After incubation for 24 h, the culture fluid was harvested and centrifuged at 1,700× *g* for 10 min. The virus suspension was stored at −80°C until use.

Antibodies

The production of rabbit polyclonal anti-NP antibody was described previously [53], and this antibody was used as a primary antibody for indirect immunofluorescence assay. A goat anti-rabbit IgG antibody conjugated to Alexa Fluor 488 or Alexa Fluor 568 was purchased from Invitrogen and used as a secondary antibody for indirect immunofluorescence assay. A polyclonal antibody against influenza A virus was obtained from 2-month-old female rabbit immunized with 250 μg of purified virions of influenza virus strain A/Puerto Rico/8/34 [54]. The generation of antibodies was boosted three times and used as neutralizing antibodies to block the influenza virus infection.

Determination of the inhibition effect of oseltamivir on virus production

MDCK cells were infected with influenza virus A/WSN/33 at a multiplicity of infection (MOI) of 0.001 PFU per cell. After virus adsorption at 37°C for 1 hour, the cells were washed with serum-free MEM and incubated at 37°C with maintenance medium (MEM containing vitamins and 0.1% BSA) containing oseltamivir. At 48 hours post infection (hpi), culture supernatant was collected, and then its viral titer was determined by plaque assays.

Generation of neuraminidase (NA)-deficient viruses

An NA-deficient influenza virus possessing the terminal sequences of NA segment but lacking the NA coding region, which was replaced with *enhanced green fluorescent protein (EGFP)* gene, was generated by reverse genetics as described previously [29,30]. For reverse genetics, we used plasmids containing cDNAs of the influenza virus A/WSN/33 viral genome under the control of the human RNA polymerase I promoter (referred to as Pol I plasmids). Briefly, 293T cells were transfected with seven Pol I plasmids for production of all vRNA segments of influenza virus A/WSN/33 and one for the mutant NA vRNA segment containing *EGFP* ORF, together with protein expression vectors for PB2, PB1, PA, and NP controlled by the chicken β-actin promoter (pCAGGS). TransIT-293 (Mirus) was used for transfection. At 24 hours post transfection, recombinant viruses were harvested from the cell surface using bacterial NA derived from *Clostridium perfringens* (sigma). MDCK cells were infected with harvested recombinant viruses treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin (1 μg/ml). After confirmation of GFP fluorescence derived from amplified recombinant virus genomes at 48 hours after infection, the recombinant viruses on the cell surface were collected using bacterial NA. The viral titer of recombinant viruses was determined by counting the number of infected foci using a fluorescence microscopy (Carl Zeiss).

Indirect immunofluorescence assay

Cells on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.2% NP-40 in PBS. The coverslips were soaked in 1% bovine serum albumin in PBS, and then incubated at room temperature for 1 hour with a primary antibody. After being washed twice with PBS, the coverslips were incubated at room temperature for 1 hour with a secondary antibody. The coverslips were then incubated at room temperature for 5 min with 3 μM 4′,6′-diamidino-2-phenylindole (DAPI) and finally mounted on glass plates, and cells were observed under the fluorescence microscope.

Live cell imaging analyses

Living cells were analyzed using BioStation ID system (GE Healthcare). Confluent MDCK cells were infected with the NA-deficient influenza virus at the multiplicity of infection (MOI) of 0.0001 in the presence or absence of 1 μg/ml TPCK-trypsin. At 24 hours post infection, culture dishes containing infected cells were set into the chamber of BioStaion ID system, which was maintained at 37°C under 5% CO_2 and 95% humidity. Then, images were acquired during next 24 hours at interval with 1 hour. The excitation wavelength was controlled by a manual filter wheel equipped with filters suitable for enhanced green fluorescence protein (EGFP).

Transwell assay

Confluent MDCK cell monolayer was prepared on transwell inserts (BD Falcon, pore size 0.4 μm) and infected with the NA-deficient influenza virus at MOI of 0.0001. After virus adsorption at 37°C for 1 hour, the cell monolayer was washed with serum-free MEM, and maintenance medium was added into both sides within the transwells. The neutralizing antibody to influenza A virus was added into the inside or the outside of transwell inserts with the maintenance medium. Subsequently, cells were incubated at 37°C for 36 hours followed by analyses using the fluorescence microscopy.

RT-PCR

*ts*53 virus has a substitution mutation from U to C at the nucleotide position of 701 in the *PA* gene. This substitution introduces an amino acid change from wild-type Leu 226 to Pro 226 and gives a defect in the viral genome replication process [48]. However, under the permissive temperature, the level of viral genome replication is no difference between wild-type and *ts*53 [47]. To discriminate the genome of wild-type and that of *ts*53, total RNA was reverse-transcribed by reverse transcriptase (TOYOBO) with PA-895-rev (5′-TTAATTTTAAGGCATC-CATCAGCAGG-3′), which is complementary to the segment 3 positive sense RNA. The cDNA was amplified by PCR using primers, PA-895-rev and PA-695-cut (5′-TCTCCCGCCA-AACTTCTCAGGCC-3′) partially corresponding to segment 3 positive sense RNA between nucleotide sequence positions 678 to 700 except for nucleotide positions 696 and 697. Since segment 3 of *ts*53 has a substitution mutation from U to C at the nucleotide position of 701, the PCR product derived from wild-type was digested by *Stu* I but not that from *ts*53. After PCR reactions, PCR

products were digested with *Stu* I and separated through PAGE. Large and small fragments derived from *ts*53 and wild-type viruses were 220 and 199 base pairs, respectively. DNA was stained with GelRed (BIOTIUM) and visualized by UV illumination.

Supporting Information

Figure S1 Formation of cell cluster caused by initial infection. MDCK cells were infected with influenza virus A/WSN/33 at moi of 0.0003 in the presence or absence of 50 μg/ml oseltamivir phosphate. After incubation for 8 and 24 h, immunofluorescence analyses were performed using anti-NP antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen). Nuclear DAPI and viral NP staining patterns are shown in blue and green, respectively. Enlarged views are shown in red borders. Scale bar, 100 μm.
(TIF)

Figure S2 The expression of GFP derived from NA-deficient influenza virus overlapped with the localization of NP. MDCK cells were infected with NA-deficient influenza viruses at MOI of 0.0001. After incubation at 37°C for 48 hours, immunofluorescence analyses were performed using anti-NP antibody. Scale bar, 100 μm.
(TIF)

Figure S3 Influenza virus A/Udorn/72 was sensitive to oseltamivir. MDCK cells were infected with influenza virus A/Udorn/72 at a MOI of 0.001 PFU per cell. At 36 hpi, the culture supernatant was collected, and then its virus titer was determined by plaque assays. Each result was represented by a value relative to that in the absence of the drug. Error bars indicate s.d. from 3 independent experiments.
(TIF)

Video S1 NA-deficient influenza virus spreads through cell-to-cell transmission. Confluent MDCK cells were infected with NA-deficient influenza virus at MOI of 0.0001 in the presence of trypsin. After incubation at 37°C for 24 hours, a single GFP-positive cell and its vicinity were traced it during the period from 24 hpi to 48 hpi at interval of 1 hour. Live cell imaging data analyses was performed by Biostation ID (GE healthcare). Scale bar, 50 μm.
(MOV)

Video S2 NA-deficient influenza virus does not spread in the absence of trypsin. Confluent MDCK cells were infected with the NA-deficient influenza virus at MOI of 0.0001 in the absence of trypsin. After incubation at 37°C for 24 hours, a single GFP-positive cell was detected, and then this cell and neighborhood cells was traced during the period from 24 hpi to 48 hpi at interval of 1 hour. Live cell imaging data analyses were performed by Biostation ID (GE healthcare). Scale bar, 50 μm.
(MOV)

Acknowledgments

We thank A. Ishihama (Hosei University) for MDCK cells, Y. Kawaoka (University of Tokyo) for 293T cells. We also thank K. Ho for his careful proofreading of the manuscript. We are grateful to A. Kawaguchi (Kitasato University and University of Tsukuba) for his valuable discussion during the course of this work.

Author Contributions

Conceived and designed the experiments: KM TH KN. Performed the experiments: KM TH. Analyzed the data: KM TH KN. Contributed reagents/materials/analysis tools: TH. Wrote the paper: KM TH KN.

References

1. Sattentau Q (2008) Avoiding the void: cell-to-cell spread of human viruses. Nat Rev Microbiol 6: 815–826.
2. Mothes W, Sherer NM, Jin J, Zhong P (2010) Virus Cell-to-Cell Transmission. J Virol 84: 8360–8368.
3. Doceul V, Hollinshead M, van der Linden L, Smith GL (2010) Repulsion of superinfecting virions: a mechanism for rapid virus spread. Science 327: 873–876.
4. Igakura T, Stinchcombe JC, Goon PK, Taylor GP, Weber JN, et al. (2003) Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton. Science 299: 1713–1716.
5. Dustin M (2003) Viral spread through protoplasmic kiss. Nat Cell Biol 5: 271–272.
6. Hübner W, McNerney GP, Chen P, Dale BM, Gordon RE, et al. (2009) Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. Science 323: 1743–1747.
7. Pais-Correia AM, Sachse M, Guadagnini S, Robbiati V, Lasserre R, et al. (2010) Biofilm-like extracellular viral assemblies mediate HTLV-1 cell-to-cell transmission at virological synapses. Nat Med 16: 83–89.
8. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, et al. (2007) Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446: 801–805.
9. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, et al. (2009) Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457: 882–886.
10. Barton ES, Forrest JC, Connolly JL, Chappell JD, Liu Y, et al. (2001) Junction adhesion molecule is a receptor for reovirus. Cell 104: 441–451.
11. Balfe P, McKeating JA (2009) The complexities of hepatitis C virus entry. J Hepatol 51: 609–611.
12. Wiley DC, Skehel JJ (1987) The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu Rev Biochem 56: 365–394.
13. Suzuki Y, Ito T, Suzuki T, Holland RE, Chambers TM, et al. (2000) Sialic acid species as a determinant of the host range of influenza A viruses. J Virol 74: 11825–11831.
14. Skehel JJ, Bayley PM, Brown EB, Martin SR, Waterfield MD, et al. (1982) Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediatedmozawa T, membrane fusion. P Natl Acad Sci Usa 79: 968–972.
15. Skehel JJ, Waterfield MD (1975) Studies on the primary structure of the influenza virus hemagglutinin. P Natl Acad Sci Usa 72: 93–97.
16. Sato M, Yoshida S, Iida K, Tomozawa T, Kido H, et al. (2003) A novel influenza A virus activating enzyme from porcine lung: purification and characterization. Biol Chem 384: 219–227.
17. Lazarowitz SG, Choppin PW (1975) Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology 68: 440–454.
18. Klenk HD, Rott R, Orlich M, Blödorn J (1975) Activation of influenza A viruses by trypsin treatment. Virology 68: 426–439.
19. Kido H, Yokogoshi Y, Sakai K, Tashiro M, Kishino Y, et al. (1992) Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. J Biol Chem 267: 13573–13579.
20. Bullough PA, Hughson FM, Skehel JJ, Wiley DC (1994) Structure of influenza haemagglutinin at the pH of membrane fusion. Nature 371: 37–43.
21. Tamm LK, Han X, Li Y, Lai AL (2002) Structure and function of membrane fusion peptides. Biopolymers 66: 249–260.
22. Air GM, Laver WG (1989) The neuraminidase of influenza virus. Proteins 6: 341–356.
23. Huang IC, Li W, Sui J, Marasco W, Choe H, et al. (2008) Influenza A virus neuraminidase limits viral superinfection. J Virol 82: 4834–4843.
24. Palese P, Tobita K, Ueda M, Compans RW (1974) Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology 61: 397–410.
25. Shibata S, Yamamoto-Goshima F, Maeno K, Hanaichi T, Fujita Y, et al. (1993) Characterization of a temperature-sensitive influenza B virus mutant defective in neuraminidase. J Virol 67: 3264–3273.
26. Gubareva LV, Nedyalkova MS, Novikov DV, Murti KG, Hoffmann E, et al. (2002) A release-competent influenza A virus mutant lacking the coding capacity for the neuraminidase active site. J Gen Virol 83: 2683–2692.
27. Nedyalkova MS, Hayden FG, Webster RG, Gubareva LV (2002) Accumulation of defective neuraminidase (NA) genes by influenza A viruses in the presence of NA inhibitors as a marker of reduced dependence on NA. J Infect Dis 185: 591–598.
28. Lew W, Chen X, Kim CU (2000) Discovery and development of GS 4104 (oseltamivir): an orally active influenza neuraminidase inhibitor. Curr Med Chem 7: 663–672.

29. Fujii Y, Goto H, Watanabe T, Yoshida T, Kawaoka Y (2003) Selective incorporation of influenza virus RNA segments into virions. P Natl Acad Sci Usa 100: 2002–2007.
30. Shinya K, Fujii Y, Ito H, Ito T, Kawaoka Y (2004) Characterization of a neuraminidase-deficient influenza a virus as a potential gene delivery vector and a live vaccine. J Virol 78: 3083–3088.
31. Timpe JM, Stamataki Z, Jennings A, Hu K, Farquhar MJ, et al. (2008) Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. Hepatology 47: 17–24.
32. Gupta P, Balachandran R, Ho M, Enrico A, Rinaldo C (1989) Cell-to-cell transmission of human immunodeficiency virus type 1 in the presence of azidothymidine and neutralizing antibody. J Virol 63: 2361–2365.
33. Pinto LH, Holsinger LJ, Lamb RA (1992) Influenza virus M2 protein has ion channel activity. Cell 69: 517–528.
34. Davies WL, Grunert RR, Haff RF, McGahen JW, Neumayer EM, et al. (1964) Antiviral Activity of 1-Adamantanamine (Amantadine). Science 144: 862–863.
35. Takeda M, Pekosz A, Shuck K, Pinto LH, Lamb RA (2002) Influenza a virus M2 ion channel activity is essential for efficient replication in tissue culture. J Virol 76: 1391–1399.
36. Chodosh J, Gan Y, Holder VP, Sixbey JW (2000) Patterned entry and egress by Epstein-Barr virus in polarized CR2-positive epithelial cells. Virology 266: 387–396.
37. Fuller S, von Bonsdorff CH, Simons K (1984) Vesicular stomatitis virus infects and matures only through the basolateral surface of the polarized epithelial cell line, MDCK. Cell 38: 65–77.
38. Schlie K, Maisa A, Freiberg F, Groseth A, Strecker T, et al. (2010) Viral protein determinants of Lassa virus entry and release from polarized epithelial cells. J Virol 84: 3178–3188.
39. Blau DM, Compans RW (1995) Entry and release of measles virus are polarized in epithelial cells. Virology 210: 91–99.
40. Brock SC, Goldenring JR, Crowe JE, Jr. (2003) Apical recycling systems regulate directional budding of respiratory syncytial virus from polarized epithelial cells. Proc Natl Acad Sci U S A 100: 15143–15148.
41. Roberts SR, Compans RW, Wertz GW (1995) Respiratory syncytial virus matures at the apical surfaces of polarized epithelial cells. J Virol 69: 2667–2673.
42. Tseng CT, Tseng J, Perrone L, Worthy M, Popov V, et al. (2005) Apical entry and release of severe acute respiratory syndrome-associated coronavirus in polarized Calu-3 lung epithelial cells. J Virol 79: 9470–9479.
43. Nayak DP, Hui EK, Barman S (2004) Assembly and budding of influenza virus. Virus Research 106: 147–165.
44. Carrasco M, Amorim MJ, Digard P (2004) Lipid raft-dependent targeting of the influenza A virus nucleoprotein to the apical plasma membrane. Traffic 5: 979–992.
45. Cresawn KO, Potter BA, Oztan A, Guerriero CJ, Ihrke G, et al. (2007) Differential involvement of endocytic compartments in the biosynthetic traffic of apical proteins. Embo J 26: 3737–3748.
46. Guerriero CJ, Lai Y, Weisz OA (2008) Differential sorting and Golgi export requirements for raft-associated and raft-independent apical proteins along the biosynthetic pathway. J Biol Chem 283: 18040–18047.
47. Kawaguchi A, Naito T, Nagata K (2005) Involvement of influenza virus PA subunit in assembly of functional RNA polymerase complexes. J Virol 79: 732–744.
48. Sugiura A, Ueda M, Tobita K, Enomoto C (1975) Further isolation and characterization of temperature-sensitive mutants of influenza virus. Virology 65: 363–373.
49. Guyader M, Kiyokawa E, Abrami L, Turelli P, Trono D (2002) Role for human immunodeficiency virus type 1 membrane cholesterol in viral internalization. J Virol 76: 10356–10364.
50. Jolly C, Sattentau QJ (2005) Human immunodeficiency virus type 1 virological synapse formation in T cells requires lipid raft integrity. J Virol 79: 12088–12094.
51. Phillips DM (1994) The role of cell-to-cell transmission in HIV infection. AIDS 8: 719–731.
52. Sato H, Orenstein J, Dimitrov D, Martin M (1992) Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. Virology 186: 712–724.
53. Kawaguchi A, Momose F, Nagata K (2011) Replication-coupled and host factor-mediated encapsidation of the influenza virus genome by viral nucleoprotein. J Virol 85: 6197–6204.
54. Watanabe K, Handa H, Mizumoto K, Nagata K (1996) Mechanism for inhibition of influenza virus RNA polymerase activity by matrix protein. J Virol 70: 241–247.

Journal of General Virology (2011), **92**, 1416–1427 DOI 10.1099/vir.0.030403-0

Characterization of influenza A viruses isolated from wild waterfowl in Zambia

Edgar Simulundu,[1] Akihiro Ishii,[1,2] Manabu Igarashi,[1] Aaron S. Mweene,[3] Yuka Suzuki,[1,2] Bernard M. Hang'ombe,[4] Boniface Namangala,[4] Ladslav Moonga,[4] Rashid Manzoor,[1] Kimihito Ito,[1] Ichiro Nakamura,[1,3] Hirofumi Sawa,[1,3] Chihiro Sugimoto,[1,3] Hiroshi Kida,[1,5,6] Chuma Simukonda,[7] Wilbroad Chansa,[7] Jack Chulu[7] and Ayato Takada[1,2]

Correspondence
Ayato Takada
atakada@czc.hokudai.ac.jp

[1]Hokkaido University Research Center for Zoonosis Control, Kita-20, Nishi-10, Kita-ku, Sapporo 001-0020, Japan

[2]Hokudai Center for Zoonosis Control in Zambia, School of Veterinary Medicine, The University of Zambia, PO Box 32379, Lusaka, Zambia

[3]Department of Disease Control, School of Veterinary Medicine, The University of Zambia, PO Box 32379, Lusaka, Zambia

[4]Department of Paraclinical Studies, School of Veterinary Medicine, The University of Zambia, PO Box 32379, Lusaka, Zambia

[5]Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[6]Japan Science and Technology Agency Basic Research Programs, Saitama, Japan

[7]Zambia Wildlife Authority, Kafue Road, Private Bag 1, Chilanga, Zambia

Although the quest to clarify the role of wild birds in the spread of the highly pathogenic H5N1 avian influenza virus (AIV) has yielded considerable data on AIVs in wild birds worldwide, information regarding the ecology and epidemiology of AIVs in African wild birds is still very limited. During AIV surveillance in Zambia (2008–2009), 12 viruses of distinct subtypes (H3N8, H4N6, H6N2, H9N1 and H11N9) were isolated from wild waterfowl. Phylogenetic analyses demonstrated that all the isolates were of the Eurasian lineage. Whilst some genes were closely related to those of AIVs isolated from wild and domestic birds in South Africa, intimating possible AIV exchange between wild birds and poultry in southern Africa, some gene segments were closely related to those of AIVs isolated in Europe and Asia, thus confirming the inter-regional AIV gene flow among these continents. Analysis of the deduced amino acid sequences of internal proteins revealed that several isolates harboured particular residues predominantly observed in human influenza viruses. Interestingly, the isolates with human-associated residues exhibited higher levels of virus replication in the lungs of infected mice and caused more morbidity as measured by weight loss than an isolate lacking such residues. This study stresses the need for continued monitoring of AIVs in wild and domestic birds in southern Africa to gain a better understanding of the emergence of strains with the potential to infect mammals.

Received 9 January 2011
Accepted 28 February 2011

INTRODUCTION

Avian influenza viruses (AIVs) are zoonotic pathogens maintained in nature mainly in wild aquatic birds (Olsen *et al.*, 2006; Webster *et al.*, 1992). Viruses of 16 different haemagglutinin (HA) (H1–H16) and nine neuraminidase (NA) (N1–N9) subtypes have been identified in waterfowl reservoirs. These viruses are usually non-pathogenic for their natural hosts. It is generally accepted that highly pathogenic AIVs (HPAIVs), particularly of the H5 and H7 subtypes, emerge from low-pathogenic AIV (LPAIV) precursors once introduced into poultry and that they may not be harboured by wild birds (Capua & Alexander, 2006; Röhm *et al.*, 1995). After emerging in China in 1996, H5N1 HPAIV spread rapidly throughout Asia, Europe, the

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this study are AB569476–AB569571.

Supplementary data on the phylogenetic relationships of AIVs from wild birds in Zambia are available with the online version of this paper.

Middle East and Africa, causing unprecedented outbreaks in wild birds, poultry and occasional human infections that have risen to pose a significant pandemic threat (Ducatez *et al.*, 2006; Li *et al.*, 2004; Smith *et al.*, 2006; Wang *et al.*, 2008). The rapid spread of the H5N1 HPAIV and the detection of H5N2 AIVs with an HP viral genotype in healthy wild waterfowl in Africa (Gaidet *et al.*, 2008) have heightened the possibility of the existence of a wild-bird reservoir for HPAIVs and underscore the need to improve our current understanding of the eco-epidemiological dynamics of AIVs in nature.

As early as 1961, Africa recorded the first outbreak of HPAIV in wild birds, which caused the death of approximately 1300 common terns (Capua & Alexander, 2006). Until 2004 when H5N2 HPAIV caused an outbreak in South African ostriches, there had been no reported cases of HP avian influenza in Africa. The continent's first experience with the Asian-origin H5N1 HPAIV was in 2006 in Nigeria (Ducatez *et al.*, 2006). The virus has since spread to several African countries, affecting a range of avian species with sporadic spillover into humans. Egypt is the African country that has recorded the highest number of human infections with the H5N1 HPAIV to date, with 115 confirmed cases, of which 38 were fatal (World Health Organization, 2010). Despite the significance of these events, which pose a serious threat to animal and public health, as well as to food security in Africa, very little is known about AIVs circulating in wild birds in Africa. Presently, there is very limited GenBank coverage (no more than three complete genomes) of non-pathogenic/LP viral genes of AIVs isolated from African wild birds.

Repeated direct transmissions of AIVs from poultry to humans and other mammals have stimulated investigations into the pathogenicity and transmission mechanisms of AIVs in mammals. Prior to the H5N1 'bird flu' incident in Hong Kong in 1997, which marked the first recorded instance of a purely AIV infecting and causing death in humans (Peiris *et al.*, 2007), investigations on the potential of AIVs from waterfowl to infect mammals, including humans, monkeys, pigs, ferrets and cats, have revealed a spectrum of replication, mostly with no significant disease signs (Beare & Webster, 1991; Hinshaw *et al.*, 1981; Kida *et al.*, 1994; Murphy *et al.*, 1982). In recent years, considerable advances have been made in elucidating the determinants of pathogenicity and adaptation of AIVs in mammals, especially for HP isolates involved in human infections (de Wit *et al.*, 2008). However, the mechanisms of pathogenicity and replicative capacity of LPAIVs isolated from wild birds in mammals are still poorly understood.

Sub-Saharan Africa where Zambia is located supports large populations of indigenous waterfowl and is an overwintering area for some Eurasian birds (Olsen *et al.*, 2006). Hitherto, no cases of H5N1 HPAIV have been recorded in southern Africa. Thus, AIV surveillance in wild birds and poultry in this region could provide timely information on the possible introduction of H5N1 HPAIV for mitigation purposes. Additionally, data obtained on LPAIVs in wild birds would expand our current understanding of the ecology and epidemiology of AIVs in this region.

During AIV surveillance conducted between 2008 and 2009 in Zambia, 12 viruses were isolated from wild waterfowl in Lochinvar National Park. Whole-genome sequencing was performed on each isolate, and bioinformatics approaches were employed to characterize the viruses genetically. Furthermore, based on genetic characterization results, we evaluated the replication and pathogenicity of some of the isolates in a mouse model.

RESULTS

Surveillance and virus isolation

AIV surveillance has been ongoing in Zambia since 2006 (Simulundu *et al.*, 2009). Between April 2008 and November 2009, a total of 3094 wild waterfowl faecal specimens were collected in Lochinvar National Park. On average, about 200 faecal specimens were collected every month except during the rainy season (December to March) when the wetland becomes inaccessible due to extreme flooding. Twelve AIVs were isolated (Table 1). Of the 12 isolates, seven were from ducks, four from geese and one from a great white pelican (*Pelecanus onocrotalus*). We identified five different HA (H3, H4, H6, H9 and H11) and NA (N1, N2, N6, N8 and N9) subtypes (Table 1). Among these subtypes, the H11N9 subtype is relatively uncommon, whilst H9N1 is a rare HA/NA combination. Currently, only ten H9N1 isolates are available in GenBank and none has been reported from Africa or Europe.

Phylogenetic analysis of the HA and NA genes

To understand the evolutionary relationships of AIVs isolated from wild birds in Zambia in detail, we sequenced the entire genome of each isolate and conducted phylogenetic analyses. To include, as much as possible, some AIV sequences of isolates from African birds in our analyses, some partial sequences were used. The HA and NA genes of all the viruses characterized in this study belonged to the Eurasian avian lineage (Figs 1 and 2 and Supplementary Figs S1 and S2, available in JGV Online). They clustered mostly with those of AIVs isolated in southern Africa. It was noted that the HA and NA genes of H11N9 viruses reported here formed a distinct sublineage within the Eurasian lineage (see Supplementary Figs S1c and S2c). In this report, only the H6 and H9 HA and the N2 and N8 NA gene trees are described in more detail, because these subtypes have been involved in avian influenza outbreaks in southern Africa.

The topology of the H6 HA phylogenetic tree conformed to that described previously by Bahl *et al.* (2009), particularly in the classification of isolates into the American and

Table 1. AIVs isolated from wild waterfowl in Zambia (2006–2009)

Host	Strain name	Abbreviation	Sampling date
Wild duck	A/duck/Zambia/02/08 (H6N2)	Zb02 (H6N2)	June 2008
	A/duck/Zambia/03/08 (H6N2)	Zb03 (H6N2)	June 2008
	A/duck/Zambia/04/08 (H3N8)	Zb04 (H3N8)	June 2008
	A/duck/Zambia/08/09 (H6N2)	Zb08 (H6N2)	August 2009
	A/duck/Zambia/10/09 (H6N2)	Zb10 (H6N2)	September 2009
	A/duck/Zambia/11/09 (H11N9)	Zb11 (H11N9)	September 2009
	A/duck/Zambia/12/09 (H11N9)	Zb12 (H11N9)	September 2009
Wild goose	A/goose/Zambia/05/08 (H3N8)	Zb05 (H3N8)	July 2008
	A/goose/Zambia/06/08 (H3N8)	Zb06 (H3N8)	July 2008
	A/goose/Zambia/07/08 (H4N6)	Zb07 (H4N6)	September 2008
	A/goose/Zambia/09/09 (H11N9)	Zb09 (H11N9)	September 2009
Wild pelican	A/pelican/Zambia/01/06 (H3N6)*	Zb01 (H3N6)	August 2006
	A/pelican/Zambia/13/09 (H9N1)	Zb13 (H9N1)	November 2009

*The first influenza virus isolate from an avian host in Zambia (Simulundu *et al.*, 2009).

Eurasian/American lineages (Fig. 1a). The H6 HA genes reported in this study belonged to a group of viruses of the Eurasian/American lineage that consisted of contemporary H6 strains isolated from wild aquatic birds in Africa, Asia and America, including those viruses that were introduced into terrestrial poultry in Taiwan and South Africa (Fig. 1a). They shared a common ancestor with an H6N8 virus that caused avian influenza in South African ostriches in 2007 (Abolnik *et al.*, 2010) (Fig. 1a). The H6 HA genes of the isolates obtained in Zambia were distinct from those that caused an avian influenza outbreak in chickens in South Africa in 2002.

Genetic and antigenic analyses of the HA genes of H9N2 AIVs have shown that these viruses separate into three main Eurasian lineages (Xu *et al.*, 2007). These lineages are represented by chicken/Beijing/1/94, quail/Hong Kong/G1/97 and duck/Hong Kong/Y439/97 (Fig. 1b). The HA gene of Zb13 (H9N1) belonged to the duck/Hong Kong/Y439/97-like lineage (also called the Korean lineage) and was most closely related to that of ostrich/South Africa/AI1586/08 (H9N2).

In the N2 NA gene tree, AIVs isolated in this study fell in a Eurasian sublineage composed of viruses isolated mainly from wild aquatic birds in Asia, Europe and Africa (Fig. 2a). They clustered together with strains isolated from an ostrich and from a wild goose in South Africa in 2008, as well as two other strains isolated in China and Japan. The H5N2 AIVs with a genotype characteristic of HPAIVs detected from wild ducks in Nigeria also belonged to this sublineage. The N2 NA phylogeny further revealed that the N2 genes of viruses that caused outbreaks of avian influenza in South Africa in 2002 were distinct from those characterized in this study, a finding that is in concordance with their HA phylogenetic comparisons (Figs 1a and 2a).

Phylogenetic analysis of the N8 NA genes showed several sublineages within the Eurasian avian lineage, namely, early 1 and 2, contemporary 1 and 2 and European gull isolates (Fig. 2b). The NA genes of Zb04 (H3N8), Zb05 (H3N8) and Zb06 (H3N8) belonged to the contemporary 1 sublineage, which consisted of AIVs isolated mostly from wild birds in Europe and southern Africa.

Phylogenetic analysis of the internal protein genes

Broadly, the topologies of the internal protein gene trees showed assortment of the AIVs into the American and Eurasian avian lineages, with early and contemporary sublineages being identifiable in the latter lineage, as described previously by Duan *et al.* (2007). The Eurasian contemporary sublineage was further divided into two to three groups.

Phylogenetic analysis of the PB2 polymerase subunit gene showed that, except for Zb04 (H3N8) which fell in group 2 of the Eurasian contemporary sublineage, all the viruses isolated in Zambia from 2006 to 2009 clustered together in group 1 (Fig. 3a). H5N1 HPAIVs also joined this group, but they were not closely related to the viruses reported here. The PB2 gene of Zb04 (H3N8) showed a close relationship to those of two H5N2 viruses isolated from domestic ducks in China.

In the PB1 polymerase subunit gene tree, three groups were observed in the Eurasian contemporary sublineage (Fig. 3b). All isolates from waterfowl in Zambia belonged to group 3. Whilst the majority of the strains isolated between 2006 and 2009 in Zambia grouped together as an independent branch, the PB1 genes of Zb04 (H3N8), Zb08 (H6N2), Zb10 (H6N2) and Zb13 (H9N1) belonged to a cluster of viruses that included two recent wild-bird isolates from South Africa, two H5N2 influenza viruses isolated from pigs in South Korea and two H5N1 HPAIVs isolated in Laos (Fig. 3b).

In the PA polymerase subunit phylogeny, all the viruses reported here belonged to group 1 and the majority of the

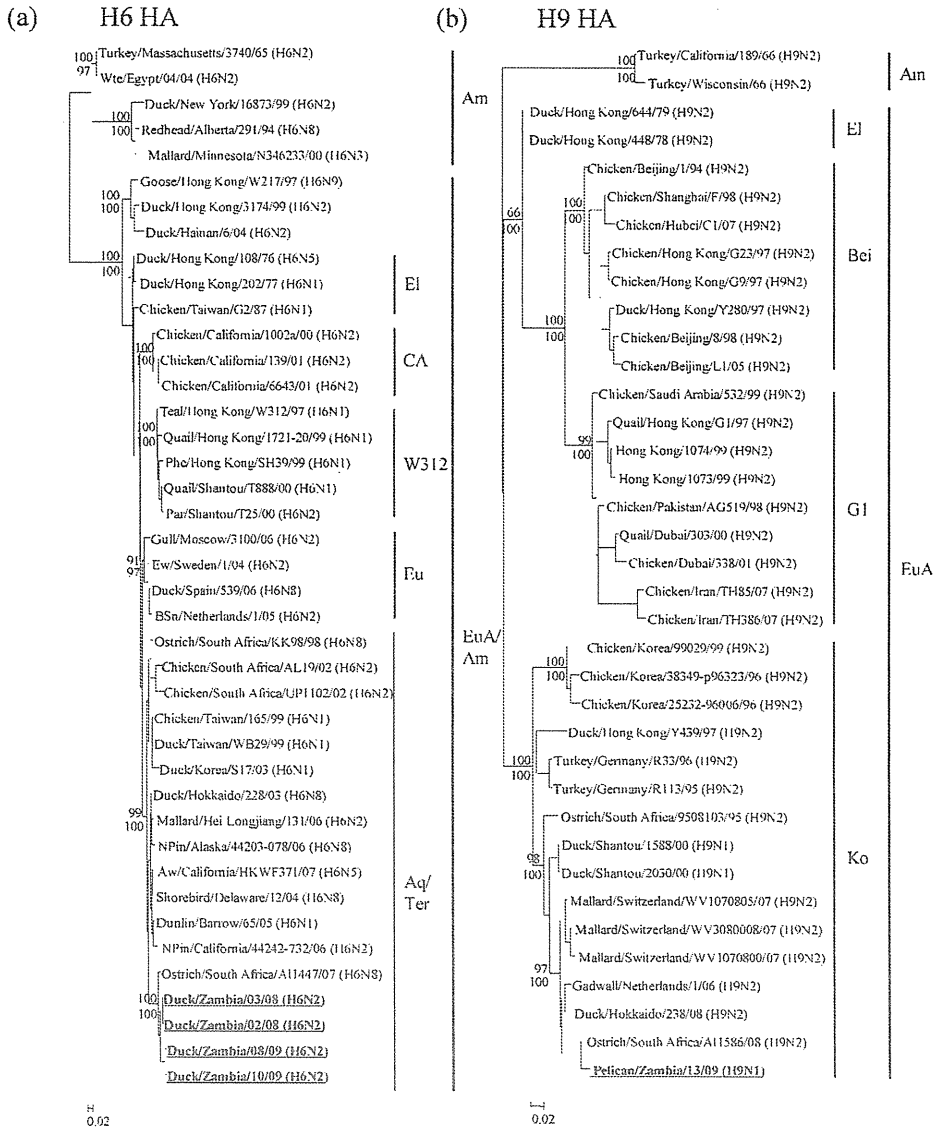

Fig. 1. Phylogenetic relationships of the H6 HA (a) and H9 HA (b) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 44–1066 (1023 bp) of H6 HA and 97–1228 (1132 bp) of H9 HA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are bold and underlined. Bars, number of substitutions per site. Lineages: Am, American; Aq/Ter, aquatic/terrestrial; Bei, chicken/Beijing/1/94-like; CA, California; El, early; Eu, Europe; EuA, Eurasian; G1, quail/Hong Kong/G1/97-like; Ko, Korean-like; W312, teal/Hong Kong/W312-like. Strain names: Aw, American wigeon; BSn, Bewick's swan; Ew, Eurasian wigeon; NPin, northern pintail; Par, partridge; Phe, pheasant; Wte, whiskered tern.

viruses grouped with those of AIVs isolated from wild birds and ostriches in South Africa (Fig. 3c). The PA gene of Zb04 (H3N8) showed a close relationship to viruses isolated from wild birds in the Netherlands. The PA gene of Zb01 (H3N6) belonged to group 2 and clustered with those of the Asian H5N1 HPAIVs, as we reported previously (Simulundu *et al.*, 2009).

The nucleoprotein (NP) gene tree showed the division of the Eurasian contemporary sublineage into three groups (Fig. 4a). All the viruses characterized in this study assorted to group 3. Eight of these strains, along with those isolated recently from wild and domestic birds in South Africa, formed a distinct cluster within this sublineage. The other four isolates clustered with viruses isolated mostly from

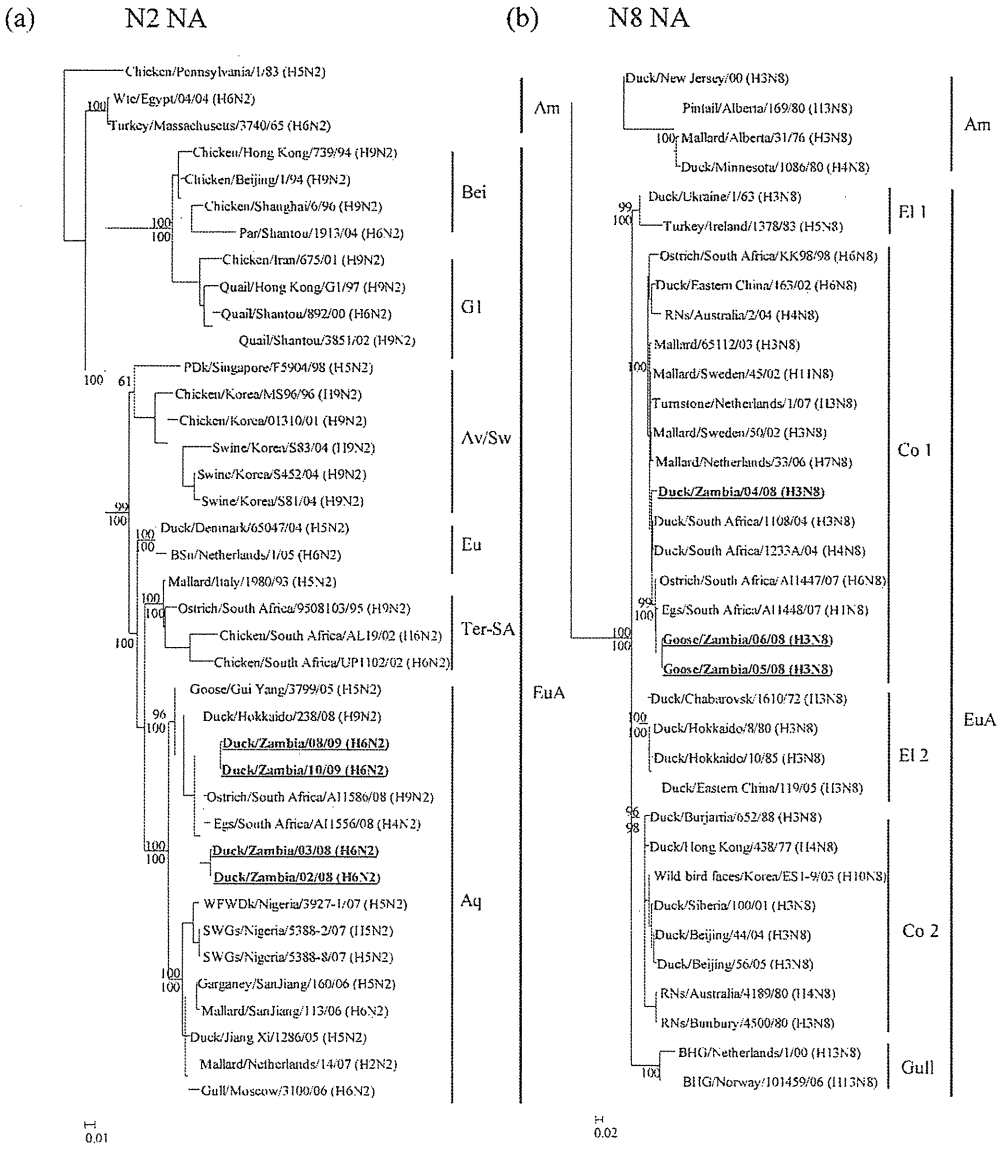

Fig. 2. Phylogenetic relationships of the N2 NA (a) and N8 NA (b) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 78–1396 (1319 bp) of N2 NA and 54–1343 (1290 bp) of N8 NA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined. Bars, number of substitutions per site. Lineages: Aq, aquatic; Av/Sw, avian/swine; Co, contemporary; Ter-SA, terrestrial, South Africa. Strain names: BHG, black-headed gull; Egs, Egyptian goose; PDk, pekin duck; RNs, red-necked stint; SWGs, spur-winged goose; WFWDk, white-faced whistling duck. Other abbreviations are listed in the legend of Fig. 1.

Europe. The NP gene of Zb01 (H3N6) was closely related to that of ostrich/South Africa/AI1447/07 (H6N8) and both these strains belonged to a group of viruses comprising early and contemporary strains.

The matrix (M) gene tree showed that all the viruses isolated from wild birds in Zambia were in group 3, but they did not all cluster together (Fig. 4b). The majority of the viruses reported in this study grouped with isolates obtained from wild and domestic birds in South Africa and appear to have been derived from A/mallard/Netherlands/1/06 (H8N4)-like viruses. The M genes of Zb01 (H3N6) and Zb04 (H3N8) were closely related to that of turkey/Italy/3620/99 (H7N1), whilst those of Zb02 (H6N2), Zb03 (H6N2), Zb05 (H3N8) and Zb06 (H3N8) grouped with that of an H9N2 virus isolated from an ostrich in South Africa.

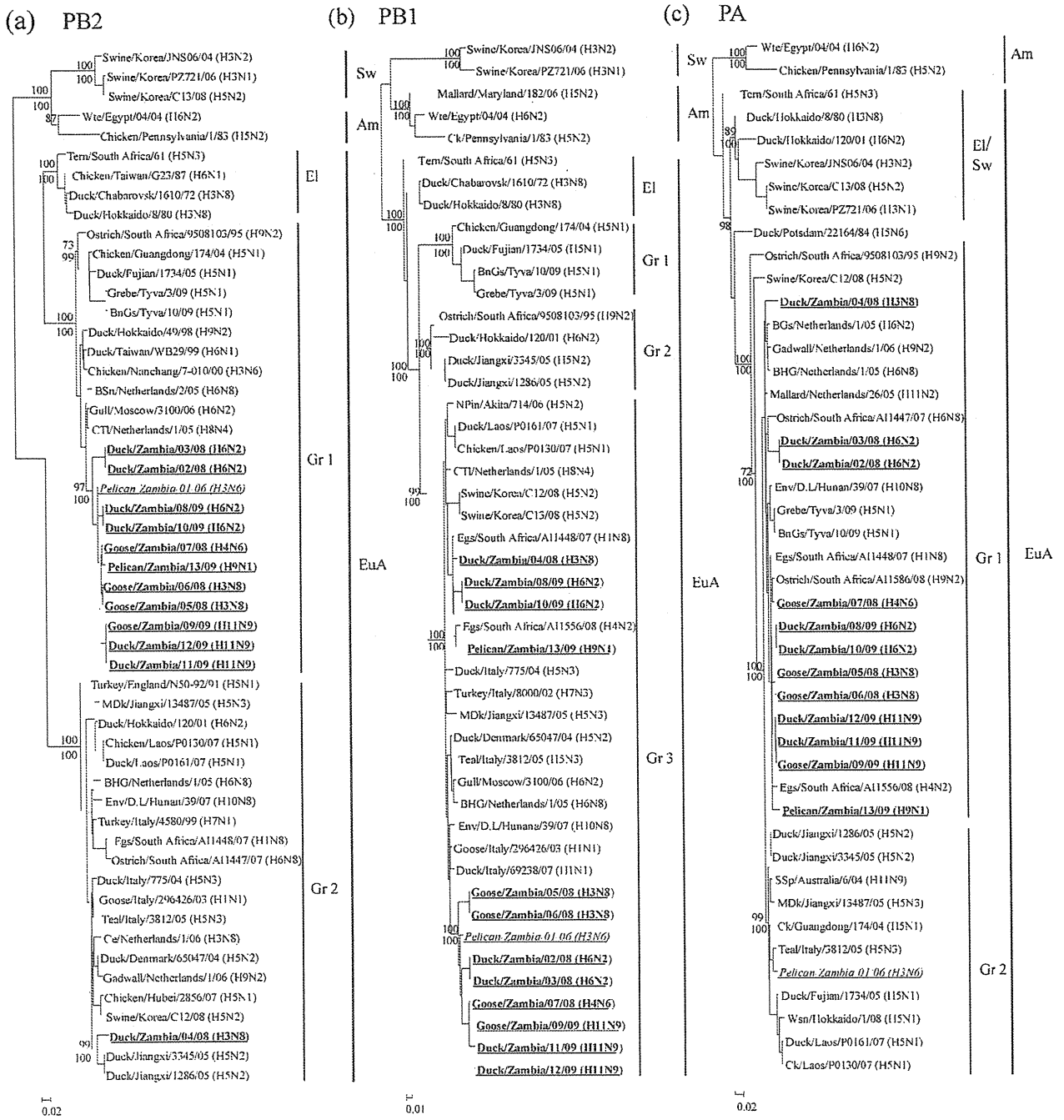

Fig. 3. Phylogenetic relationships of the PB2 (a), PB1 (b) and PA (c) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 56–2285 (2230 bp) of PB2, 64–2281 (2218 bp) of PB1 and 30–2098 (2069 bp) of PA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Due to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined, whilst the first AIV isolate in Zambia is italicized and underlined. Bars, number of substitutions per site. Lineages: El/Sw, early/swine; Sw, swine. Strain names: BGs, barnacle goose; BnGs, bean goose; Ce, common eider; Ck, chicken; CTl, common teal; D.L, Dongting Lake; Env, environment; MDk, migratory duck; SSp, sharp-tailed sandpiper; Wsn, whooper swan. Other abbreviations are listed in the legends of Figs 1 and 2.

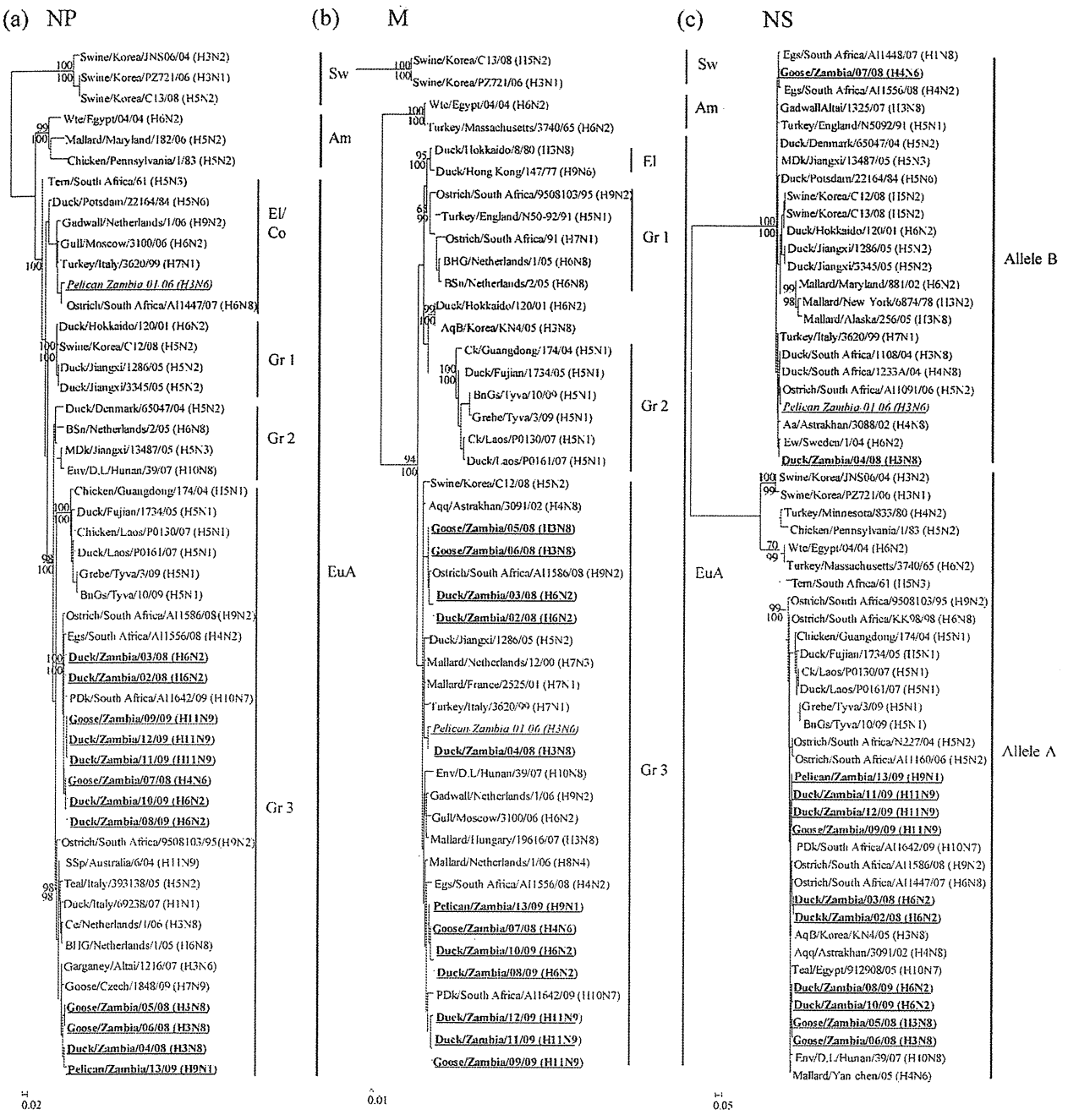

Fig. 4. Phylogenetic relationships of the NP (a), M (b) and NS (c) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 46–1489 (1444 bp) of NP, 32–753 (722 bp) of M and 57–705 (649 bp) of NS. Numbers above and below the branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined, whilst the first influenza virus isolate from an avian host in Zambia is italicized. Bars, number of substitutions per site. Lineages: El/Co, early/contemporary. Strain names: Aa, *Anas angustirostris*; AqB, aquatic bird; Aqq, *Anas querquedula*. Other abbreviations are listed in the legends of Figs 1–3.

Phylogenetic analysis of the non-structural (NS) gene indicated that the NS genes of ten of the viruses from wild birds in Zambia comprised the A allele, whilst the other three were of the B allele (Fig. 4c). Some of the NS genes were closely related to viruses isolated mainly in Asia and Africa, particularly those isolated in South Africa (Fig. 4c).

The NS gene tree clearly demonstrated that, among the viruses examined, the A allele was predominant and that two genetically distinct gene pools, corresponding to NS alleles A and B, were co-circulating in wild birds in this region during the surveillance period.

Amino acid sequence analysis

Although it is difficult to ascertain the capacity of a non-pathogenic AIV/LPAIV from wild waterfowl to cause interspecies transmission into other animals, close monitoring of host-associated signatures in viral proteins may provide some clues regarding an isolate's zoonotic potential. Several amino acids that are preferentially associated with human influenza viruses have been described (Chen *et al.*, 2006; Finkelstein *et al.*, 2007; Shaw *et al.*, 2002). We examined the deduced amino acid sequences of all the internal proteins of all the wild-bird isolates from Zambia and identified some human-associated amino acids in the genome of some strains (Table 2). Zb08 (H6N2) and Zb10 (H6N2) possessed the human-associated amino acid methionine at position 475 of the PB2 protein, which has been described to be 100% conserved in the influenza viruses that caused the 1918, 1957 and 1968 human pandemics (Finkelstein *et al.*, 2007). Zb13 (H9N1) had a serine at position 66 of the PB1-F2 polypeptide, which was shown previously to contribute to increased virulence in mice (Conenello *et al.*, 2007). Zb13 (H9N1) also possessed the human-associated amino acid alanine at position 76 of the PB1-F2 protein. Six isolates were found to have the human-associated amino acid serine at position 82 of the PB1-F2 protein, whilst only Zb08 (H6N2) and Zb10 (H6N2) had the human-associated amino acid glycine at position 87 of this polypeptide. In the M2 protein, Zb04 (H3N8) possessed the human-associated amino acid valine at position 28. At position 55 of the M2 protein, Zb07 (H4N6), Zb08 (H6N2), Zb10 (H6N2) and Zb12 (H11N9) were found to possess the human-associated amino acid phenylalanine. It is noteworthy that, although the human-associated amino acids found in some of the virus isolates analysed in this report are not unique to these isolates, these residues are rarely found among AIVs isolated from members of the orders Anseriformes and Charadriiformes (our unpublished data).

Table 2. Human-associated amino acids identified in viral proteins of AIVs isolated in Zambia

Protein	Aa position*	Host		Isolate†
		Avian	Human	
PB2	475	L	M	Zb08 (H6N2)
				Zb10 (H6N2)
PB1-F2	66	N	S‡	Zb13 (H9N1)
	76	V	A	Zb13 (H9N1)
	82	L	S	Zb04 (H3N8)
				Zb05 (H3N8)
				Zb06 (H3N8)
				Zb08 (H6N2)
				Zb10 (H6N2)
				Zb13 (H9N1)
	87	E	G	Zb08 (H6N2)
				Zb10 (H6N2)
M2	28	I	V	Zb04 (H3N8)
	55	L	F	Zb07 (H4N6)
				Zb08 (H6N2)
				Zb10 (H6N2)
				Zb12 (H11N9)

*For references of human-associated residues at these specific positions, see Chen *et al.* (2006), Finkelstein *et al.* (2007) and Shaw *et al.* (2002).

†Names of isolates possessing human-associated amino acid residues.

‡The amino acid serine at position 66 of the PB1-F2 protein is not a human-associated residue but was shown previously to increase virulence in mice (Conenello *et al.*, 2007).

Replication and pathogenicity of selected viruses in mice

Amino acid sequence analysis revealed that several isolates from wild birds in Zambia had human-associated residues in their genome (Table 2). Therefore, we sought to investigate whether there could be a difference in virus replication and/or pathogenicity in a mammalian host between viruses either possessing or lacking human-associated residues. For this purpose, we compared the replication ability and pathogenicity of two isolates, Zb03 (H6N2) and Zb10 (H6N2), in mice. Four human-associated residues were identified in some viral proteins of Zb10 (H6N2), whilst none was observed in the genome of Zb03 (H6N2) (Table 2). We also tested the replication capacity and pathogenicity of Zb04 (H3N8) in mice, because it had two human-associated residues in its genome and was of a subtype distinct from that of Zb10 (H6N2).

All the tested viruses replicated in the lungs of mice without prior adaptation, with virus titres ranging from $10^{3.3}$ to $10^{4.8}$ EID_{50} g^{-1} (Table 3). None of the viruses was detected in the brain. It was noted that mice inoculated with Zb10 (H6N2) showed higher virus titres that were statistically significantly different from those of Zb03 (H6N2)-infected mice (Table 3). Virus was detected in the lungs of all five mice inoculated with Zb04 (H3N8) and Zb10 (H6N2), whilst, in Zb03 (H6N2)-inoculated mice, virus was detected in three of the five mice.

Mice infected with Zb10 (H6N2) exhibited more weight loss and delayed weight gain [weight returned to baseline after day 7 post-inoculation (p.i.)] than those inoculated with Zb03 (H6N2) (Fig. 5). Zb04 (H3N8)-inoculated mice showed significant weight loss early on p.i. when compared with Zb03 (H6N2)- or mock-inoculated control mice (Fig. 5). Mild to considerable ruffled fur was noted between days 1 and 3 p.i. in mice infected with Zb10 (H6N2) and Zb04 (H3N8) but not in Zb03 (H6N2)-inoculated mice.

Table 3. Replication of selected AIVs isolated from wild waterfowl in Zambia in BALB/c mice

Virus	No. positive/ total	Mean virus titre of positive samples (log_{10} EID_{50} g^{-1})		*P* value
		Lung	Brain	
Zb03 (H6N2)*	3/5	3.3	$<10^{1.5}$	–
Zb04 (H3N8)†	5/5	3.6	$<10^{1.5}$	0.099
Zb10 (H6N2)†	5/5	4.8	$<10^{1.5}$	0.001‡

*Virus with no apparent human/mammalian-associated residues in its genome.
†Viruses with human-associated residues in their genome.
‡Virus titre in the lungs of mice inoculated with Zb10 (H6N2) was significantly higher than that of Zb03 (H6N2)-inoculated mice (Student's *t*-test, $P<0.05$).

All the mice survived the infection for the 14-day observation period.

DISCUSSION

In this study, we genetically and biologically characterized AIVs isolated from wild birds in Zambia. During the surveillance period, AIVs were isolated mainly between June and November, a time frame encompassing the period when palearctic migrants are absent or rare, as well as when they are present. Palearctic birds usually start to arrive in Zambia between September and December and leave between January and May. Our isolation of AIVs between June and August of 2008 and 2009 when palearctic migrants were scarce raises the possibility of yearly persistence of AIVs in indigenous waterfowl in southern Africa. This idea is further supported by our phylogenetic analyses, which showed the separate clustering of southern African isolates, with the glycoprotein genes of H11N9 viruses characterized in this report forming a distinct sublineage within the Eurasian lineage (Fig. 3a, b and Supplementary Figs S1c and S2c). In neighbouring Zimbabwe, AIVs were also detected in Afro-tropical waterfowl in periods when palearctic birds were rare (Caron *et al.*, 2010). Moreover, AIVs were detected from Afro-tropical bird species in several major wetlands in Africa (Gaidet *et al.*, 2007). These data not only support the notion of a possible endemicity of AIVs in Afro-tropical ecosystems where high temperatures experienced in these regions may restrict the persistence and transmissibility of AIVs (Brown *et al.*, 2009), but also raise the possibility that palearctic migrants may also carry AIVs from Africa into Eurasia. However, the extent to which Afro-tropical ecosystems depend on introductions of AIVs by Eurasian migrants to sustain the possible endemic state remains to be clarified.

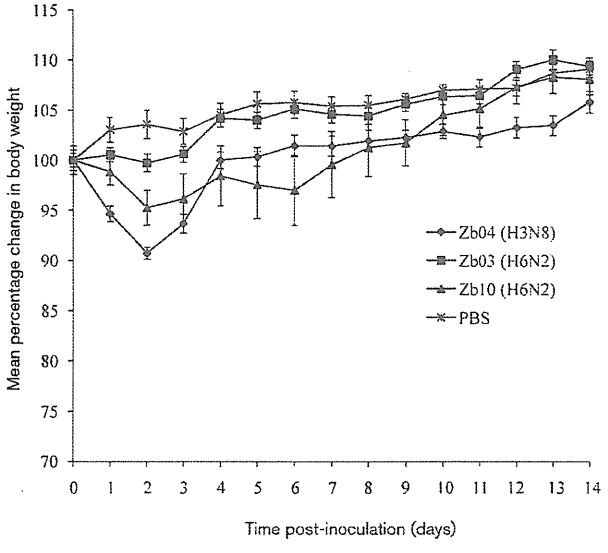

Fig. 5. Weight loss in mice inoculated with selected AIVs from wild waterfowl in Zambia. Data are presented as mean body weight change per group ±SD. Statistically significant weight loss (Student's *t*-test, $P<0.05$) was observed in Zb04 (H3N8)-inoculated mice at days 1 ($P=0.03$), 2 ($P=0.01$) and 3 ($P=0.03$) p.i. compared with mock-inoculated control mice.

The detection of five distinct HA and NA subtypes suggested that a variety of subtypes could be circulating in wild birds in this region. Whilst 11 of the isolates were detected in wild ducks and geese, confirming the major role of these birds in the perpetuation of AIVs (Olsen *et al.*, 2006; Webster *et al.*, 1992), Zb13 (H9N1) was isolated from an atypical avian host, a great white pelican. Despite several AIV surveillance studies that involved sampling from the Pelecaniformes worldwide (Gaidet *et al.*, 2007; Munster *et al.*, 2007; Olsen *et al.*, 2006), the number of AIVs detected from this order has remained low. Thus, we consider the two instances in which we isolated AIVs from these birds as incidental findings, but we do not exclude the possibility that white pelicans, which are native to southern Africa, may also play a major role in influenza virus ecology in this region.

Phylogenetic analyses demonstrated that all the gene segments of the viruses reported in this study clustered with contemporary viruses of the Eurasian avian lineage. Most genes were closely related to those of AIVs isolated from wild and domestic birds in South Africa. AIVs originating in wild birds have been implicated in avian influenza outbreaks in farmed birds in South Africa with serious economic consequences (Abolnik, 2007; Abolnik *et al.*, 2007, 2010; Alexander, 2007; Brown, 2010). These data highlight the need for continued monitoring of AIVs in wild and domestic birds in southern Africa for avian influenza control. It is also important to clarify the extent of influenza virus exchange between wild birds and domesticated birds (including ostriches) in the region, as

results from a study in China demonstrated that a two-way transmission of influenza viruses between terrestrial and aquatic birds may increase opportunities for the generation of reassortant viruses with pandemic potential (Li *et al.*, 2003). Furthermore, the potential role of human related activities (e.g. the poultry trade) in AIV dissemination should not be ignored.

A number of human-associated amino acids were observed in some viral proteins of some viruses tested. The two possible means by which AIVs may acquire 'novel' amino acids are either through genetic reassortment or through point mutations. Genetic analyses of AIVs isolated from wild and terrestrial birds in southern Africa have demonstrated the involvement of ostriches in the evolution and epidemiology of AIVs in this region (Abolnik, 2007; Abolnik *et al.*, 2007, 2010; this study). Recently, Shinya *et al.* (2009) demonstrated that ostriches may be involved in the emergence of viruses possessing mammalian-associated amino acids lysine and asparagine at positions 627 and 701 of the PB2 protein, respectively. Indeed, an examination of PB2 gene sequences of viruses isolated from ostriches in South Africa between 1995 and 2008 showed that four viruses had lysine and one virus possessed asparagine at positions 627 and 701 of the PB2 protein, respectively (data not shown). Therefore, if a two-way transmission of AIVs between ostriches and wild aquatic birds in southern Africa exists, these data indicate that the human-associated amino acids observed in some internal proteins of some of the isolates examined here may have been acquired through genetic reassortment with viruses from ostriches. Unfortunately, the lack of complete internal protein gene sequences of isolates from ostriches in South Africa for a comprehensive study makes it difficult to reach this conclusion. Moreover, genetic analyses of the deduced amino acids revealed that the surface proteins of the viruses listed in Table 2 maintained typical features of non-pathogenic wild waterfowl isolates, including conservation of putative glycosylation sites and no NA stalk deletions, and did not exhibit evidence for accelerated or increased amino acid substitutions, suggesting that these viruses may not have circulated extensively in land-based avian species. These observations leave open the possibility that the human-associated amino acids in the viral proteins of some isolates from Zambia may have been acquired in wild waterfowl or other non-gallinaceous birds. Whether some African waterfowl may provide an environment that may lead to the selection of AIVs with human/mammalian-associated amino acids is a question deserving further exploration.

In a mouse model, we demonstrated that all the tested viruses replicated in mouse lung without prior adaptation and that mice infected with isolates having human-associated residues displayed increased virus titres and caused increased morbidity, as measured by weight loss, than those inoculated with Zb03 (H6N2). Although it is tempting to conclude that possession of human-associated residues may have impacted on virus replication and pathogenicity in mice, there is need for caution, because the influence of other residues was not ruled out in the current study. In fact, there were 68 amino acid differences in viral proteins between Zb03 (H6N2) and Zb10 (H6N2). Therefore, investigations employing reverse genetics and site-directed mutagenesis may be needed to explain more fully the observed differences. To our knowledge, the present study is the first to demonstrate the ability of non-HPAIVs from wild birds in Africa to replicate without adaptation and cause illness in a mammalian host. Elsewhere, although few in number, AIVs from wild birds of considerable numbers of HA subtypes have been shown to replicate in mice and ferrets without adaptation, causing varied degrees of morbidity (Driskell *et al.*, 2010; Gillim-Ross *et al.*, 2008; Joseph *et al.*, 2007; Kim *et al.*, 2010; Wan *et al.*, 2008). These studies have highlighted the potential risk of direct transmission of non-HPAIVs from wild birds to mammalian species. Whilst direct transmission of AIVs from wild birds to humans has not been reported, serological evidence of AIV infection in three persons with substantial exposure to wild waterfowl and game birds argues for a possible direct transmission of AIVs from wild birds to humans (Gill *et al.*, 2006). Moreover, both natural and experimental infections of humans with AIVs, together with serological data, have emphasized the susceptibility of humans to several AIV subtypes (Myers *et al.*, 2007; Peiris *et al.*, 2007; Shortridge, 1992). Thus, the potential threat posed to both animal and public health by some of the viruses characterized currently cannot be overemphasized.

Here, we demonstrated that the 12 influenza viruses isolated from wild waterfowl in Zambia belonged to the contemporary Eurasian avian lineage. We have shown the possibility that AIVs could persist in wild waterfowl in a Zambian ecosystem, with transmission of viruses involving wild and domestic avian species in southern Africa, Europe and Asia. This study further established that some AIVs from wild waterfowl in Zambia may have the potential to infect mice directly without adaptation. Overall, the present study raises concerns for continued monitoring of AIVs in wild and domestic birds in southern Africa and suggests that complete characterization of isolates may help in the identification of strains that may have potential for future incursions into humans and other animals.

METHODS

Viruses and sequencing. The viruses characterized in the present study were isolated from wild waterfowl faecal specimens collected in Lochinvar National Park between April 2008 and November 2009 (Table 1). All virus isolation was performed using 10–11-day-old embryonated chicken's eggs. The isolates were subtyped by standard HA inhibition and NA inhibition tests, as well as by sequencing of the HA and NA genes. The viruses were passaged once in eggs before being used in this study. Viral RNA extraction, cDNA synthesis, PCR and sequencing were carried out as described previously (Simulundu *et al.*, 2009).

Phylogenetic analyses. Phylogenetic trees were constructed by the neighbour-joining bootstrap method with 1000 replicates applied

using MEGA4 (Tamura *et al.*, 2007). The gene tree topologies obtained in MEGA4 were then confirmed using Bayesian methods implemented in MRBAYES version 3.1.2 (Huelsenbeck & Ronquist, 2001). Specifically, we used the program ModelTest version 3.7 (Posada & Crandall, 2001), applied in PAUP* version 4.0 (Swofford, 2001), to determine the appropriate evolutionary model that best fitted the data. The HA, NA, PB2, PB1, PA and NP nucleotide sequence data were best fitted by the general time reversible plus invariant sites plus gamma-distributed (GTR+I+G) model, whilst the Hasegawa–Kishino–Yano (plus invariant sites) plus gamma-distributed models (HKY+G and HKY+I+G) were preferred for the NS and M sequence data, respectively. In Bayesian analysis, we used one to four replicates of 1 million generations, with four chains sampled every 100 generations. All replicates converged with less than 0.01 SD of split frequencies.

Experimental infection of mice. Groups of 6-week-old BALB/c mice (ten mice per group) were lightly anaesthetized with isoflurane and inoculated intranasally with 0.05 ml virus-infected chorioallantoic fluid containing Zb03 (H6N2), Zb04 (H3N8) or Zb10 (H6N2) ($10^{7.5}$ EID_{50} ml^{-1}). To serve as a control, a group of five mice was mock infected with sterile PBS. Mice were observed daily for morbidity (weight loss, ruffled fur and hunching) and mortality for 14 days. On day 3 p.i., half of the virus-inoculated mice were euthanized, and the titres of virus in the lung and brain were determined using eggs. Briefly, a 10% lung and brain tissue homogenate was prepared using minimal essential medium (Gibco) containing antibiotics. The tissue homogenates were clarified by centrifugation and titrated in 10–11-day-old embryonated chicken's eggs. The virus titre was calculated as the $\log_{10}$ EID_{50} (g tissue)$^{-1}$ by the method of Reed & Muench (1938).

ACKNOWLEDGEMENTS

We thank the Zambia Wildlife Authority for supporting the wild-bird influenza A virus surveillance programme in Zambia. We are also grateful to H. Miyamoto, A. Ohnuma and A. Yokoyama for excellent technical assistance. This work was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases and the Global COE Program 'Establishment of International Collaboration Centers for Zoonosis Control' from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

REFERENCES

Abolnik, C. (2007). Molecular characterization of H5N2 avian influenza viruses isolated from South African ostriches in 2006. *Avian Dis* **51**, 873–879.

Abolnik, C., Bisschop, S., Gerdes, T., Olivier, A. & Horner, R. (2007). Outbreaks of avian influenza H6N2 viruses in chickens arose by a reassortment of H6N8 and H9N2 ostrich viruses. *Virus Genes* **34**, 37–45.

Abolnik, C., Gerdes, G. H., Sinclair, M., Ganzevoort, B. W., Kitching, J. P., Burger, C. E., Romito, M., Dreyer, M., Swanepoel, S. & other authors (2010). Phylogenetic analysis of influenza A viruses (H6N8, H1N8, H4N2, H9N2, H10N7) isolated from wild birds, ducks, and ostriches in South Africa from 2007 to 2009. *Avian Dis* **54** (Suppl.), 313–322.

Alexander, D. J. (2007). Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. *Avian Dis* **51** (Suppl.), 161–166.

Bahl, J., Vijaykrishna, D., Holmes, E. C., Smith, G. J. D. & Guan, Y. (2009). Gene flow and competitive exclusion of avian influenza A virus in natural reservoir hosts. *Virology* **390**, 289–297.

Beare, A. S. & Webster, R. G. (1991). Replication of avian influenza viruses in humans. *Arch Virol* **119**, 37–42.

Brown, I. H. (2010). Summary of avian influenza activity in Europe, Asia, and Africa, 2006–2009. *Avian Dis* **54** (Suppl.), 187–193.

Brown, J. D., Goekjian, G., Poulson, R., Valeika, S. & Stallknecht, D. E. (2009). Avian influenza virus in water: infectivity is dependent on pH, salinity and temperature. *Vet Microbiol* **136**, 20–26.

Capua, I. & Alexander, D. J. (2006). The challenge of avian influenza to the veterinary community. *Avian Pathol* **35**, 189–205.

Caron, A., Abolnik, C., Mundava, J., Gaidet, N., Burger, C. E., Mochotlhoane, B., Bruinzeel, L., Chiweshe, N., de Garine-Wichatitsky, M. & Cumming, G. S. (2010). Persistence of low pathogenic avian influenza virus in waterfowl in a Southern African ecosystem. *EcoHealth* (Epub ahead of print).

Chen, G.-W., Chang, S.-C., Mok, C.-K., Lo, Y.-L., Kung, Y.-N., Huang, J.-H., Shih, Y.-H., Wang, J.-Y., Chiang, C. & other authors (2006). Genomic signatures of human versus avian influenza A viruses. *Emerg Infect Dis* **12**, 1353–1360.

Conenello, G. M., Zamarin, D., Perrone, L. A., Tumpey, T. & Palese, P. (2007). A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. *PLoS Pathog* **3**, 1414–1421.

de Wit, E., Kawaoka, Y., de Jong, M. D. & Fouchier, R. A. (2008). Pathogenicity of highly pathogenic avian influenza virus in mammals. *Vaccine* **26** (Suppl. 4), D54–D58.

Driskell, E. A., Jones, C. A., Stallknecht, D. E., Howerth, E. W. & Tompkins, S. M. (2010). Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. *Virology* **399**, 280–289.

Duan, L., Campitelli, L., Fan, X. H., Leung, Y. H., Vijaykrishna, D., Zhang, J. X., Donatelli, I., Delogu, M., Li, K. S. & other authors (2007). Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. *J Virol* **81**, 7529–7539.

Ducatez, M. F., Olinger, C. M., Owoade, A. A., De Landtsheer, S., Ammerlaan, W., Niesters, H. G. M., Osterhaus, A. D. M. E., Fouchier, R. A. M. & Muller, C. P. (2006). Avian flu: multiple introductions of H5N1 in Nigeria. *Nature* **442**, 37.

Finkelstein, D. B., Mukatira, S., Mehta, P. K., Obenauer, J. C., Su, X., Webster, R. G. & Naeve, C. W. (2007). Persistent host markers in pandemic and H5N1 influenza viruses. *J Virol* **81**, 10292–10299.

Gaidet, N., Dodman, T., Caron, A., Balança, G., Desvaux, S., Goutard, F., Cattoli, G., Lamarque, F., Hagemeijer, W. & Monicat, F. (2007). Avian influenza viruses in water birds, Africa. *Emerg Infect Dis* **13**, 626–629.

Gaidet, N., Cattoli, G., Hammoumi, S., Newman, S. H., Hagemeijer, W., Takekawa, J. Y., Cappelle, J., Dodman, T., Joannis, T. & other authors (2008). Evidence of infection by H5N2 highly pathogenic avian influenza viruses in healthy wild waterfowl. *PLoS Pathog* **4**, e1000127.

Gill, J. S., Webby, R., Gilchrist, M. J. R. & Gray, G. C. (2006). Avian influenza among waterfowl hunters and wildlife professionals. *Emerg Infect Dis* **12**, 1284–1286.

Gillim-Ross, L., Santos, C., Chen, Z., Aspelund, A., Yang, C.-F., Ye, D., Jin, H., Kemble, G. & Subbarao, K. (2008). Avian influenza H6 viruses productively infect and cause illness in mice and ferrets. *J Virol* **82**, 10854–10863.

Hinshaw, V. S., Webster, R. G., Easterday, B. C. & Bean, W. J., Jr (1981). Replication of avian influenza A viruses in mammals. *Infect Immun* **34**, 354–361.

Huelsenbeck, J. P. & Ronquist, F. R. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.

Joseph, T., McAuliffe, J., Lu, B., Jin, H., Kemble, G. & Subbarao, K. (2007). Evaluation of replication and pathogenicity of avian influenza a H7 subtype viruses in a mouse model. *J Virol* **81**, 10558–10566.

Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K. F., Kawaoka, Y. & Webster, R. G. (1994). Potential for transmission of avian influenza viruses to pigs. *J Gen Virol* **75**, 2183–2188.

Kim, H.-R., Lee, Y.-J., Lee, K.-K., Oem, J.-K., Kim, S.-H., Lee, M. -H., Lee, O.-S. & Park, C.-K. (2010). Genetic relatedness of H6 subtype avian influenza viruses isolated from wild birds and domestic ducks in Korea and their pathogenicity in animals. *J Gen Virol* **91**, 208–219.

Li, K. S., Xu, K. M., Peiris, J. S., Poon, L. L., Yu, K. Z., Yuen, K. Y., Shortridge, K. F., Webster, R. G. & Guan, Y. (2003). Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans? *J Virol* **77**, 6988–6994.

Li, K. S., Guan, Y., Wang, J., Smith, G. J. D., Xu, K. M., Duan, L., Rahardjo, A. P., Puthavathana, P., Buranathai, C. & other authors (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* **430**, 209–213.

Munster, V. J., Baas, C., Lexmond, P., Waldenström, J., Wallensten, A., Fransson, T., Rimmelzwaan, G. F., Beyer, W. E., Schutten, M. & other authors (2007). Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *PLoS Pathog* **3**, e61.

Murphy, B. R., Hinshaw, V. S., Sly, D. L., London, W. T., Hosier, N. T., Wood, F. T., Webster, R. G. & Chanock, R. M. (1982). Virulence of avian influenza A viruses for squirrel monkeys. *Infect Immun* **37**, 1119–1126.

Myers, K. P., Setterquist, S. F., Capuano, A. W. & Gray, G. C. (2007). Infection due to 3 avian influenza subtypes in United States veterinarians. *Clin Infect Dis* **45**, 4–9.

Olsen, B., Munster, V. J., Wallensten, A., Waldenström, J., Osterhaus, A. D. & Fouchier, R. A. (2006). Global patterns of influenza A virus in wild birds. *Science* **312**, 384–388.

Peiris, J. S., de Jong, M. D. & Guan, Y. (2007). Avian influenza virus (H5N1): a threat to human health. *Clin Microbiol Rev* **20**, 243–267.

Posada, D. & Crandall, K. A. (2001). Selecting the best-fit model of nucleotide substitution. *Syst Biol* **50**, 580–601.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493–497.

Röhm, C., Horimoto, T., Kawaoka, Y., Süss, J. & Webster, R. G. (1995). Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? *Virology* **209**, 664–670.

Shaw, M., Cooper, L., Xu, X., Thompson, W., Krauss, S., Guan, Y., Zhou, N., Klimov, A., Cox, N. & other authors (2002). Molecular changes associated with the transmission of avian influenza a H5N1 and H9N2 viruses to humans. *J Med Virol* **66**, 107–114.

Shinya, K., Makino, A., Ozawa, M., Kim, J. H., Sakai-Tagawa, Y., Ito, M., Le, Q. M. & Kawaoka, Y. (2009). Ostrich involvement in the selection of H5N1 influenza virus possessing mammalian-type amino acids in the PB2 protein. *J Virol* **83**, 13015–13018.

Shortridge, K. F. (1992). Pandemic influenza: a zoonosis? *Semin Respir Infect* **7**, 11–25.

Simulundu, E., Mweene, A. S., Tomabechi, D., Hang'ombe, B. M., Ishii, A., Suzuki, Y., Nakamura, I., Sawa, H., Sugimoto, C. & other authors (2009). Characterization of H3N6 avian influenza virus isolated from a wild white pelican in Zambia. *Arch Virol* **154**, 1517–1522.

Smith, G. J. D., Fan, X. H., Wang, J., Li, K. S., Qin, K., Zhang, J. X., Vijaykrishna, D., Cheung, C. L., Huang, K. & other authors (2006). Emergence and predominance of an H5N1 influenza variant in China. *Proc Natl Acad Sci U S A* **103**, 16936–16941.

Swofford, D. L. (2001). PAUP*: phylogenetic analysis using parsimony (and other methods) 4.0 beta. Sunderland, MA: Sinauer Associates.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Wan, H., Sorrell, E. M., Song, H., Hossain, M. J., Ramirez-Nieto, G., Monne, I., Stevens, J., Cattoli, G., Capua, I. & other authors (2008). Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. *PLoS ONE* **3**, e2923.

Wang, G., Zhan, D., Li, L., Lei, F., Liu, B., Liu, D., Xiao, H., Feng, Y., Li, J. & other authors (2008). H5N1 avian influenza re-emergence of Lake Qinghai: phylogenetic and antigenic analyses of the newly isolated viruses and roles of migratory birds in virus circulation. *J Gen Virol* **89**, 697–702.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* **56**, 152–179.

World Health Organization (2010). Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_12_29/en/index.html. Accessed 4 January 2011.

Xu, K. M., Smith, G. J. D., Bahl, J., Duan, L., Tai, H., Vijaykrishna, D., Wang, J., Zhang, J. X., Li, K. S. & other authors (2007). The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. *J Virol* **81**, 10389–10401.

SUPPLEMENT ARTICLE

Antibody-Dependent Enhancement of Marburg Virus Infection

Eri Nakayama,[1] Daisuke Tomabechi,[1] Keita Matsuno,[1] Noriko Kishida,[2] Reiko Yoshida,[1] Heinz Feldmann,[3] and Ayato Takada[1]

[1]Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, [2]Laboratory of Influenza Virus Surveillance, Center for Influenza Virus Research, National Institute of Infectious Diseases, Tokyo, Japan; and [3]Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, Montana

Background. Marburg virus (MARV) and Ebola virus (EBOV) cause severe hemorrhagic fever in primates. Earlier studies demonstrated that antibodies to particular epitopes on the glycoprotein (GP) of EBOV enhanced virus infectivity in vitro.

Methods. To investigate this antibody-dependent enhancement (ADE) in MARV infection, we produced mouse antisera and monoclonal antibodies (mAbs) to the GPs of MARV strains Angola and Musoke.

Results. The infectivity of vesicular stomatitis virus pseudotyped with Angola GP in K562 cells was significantly enhanced in the presence of Angola GP antisera, whereas only minimal ADE activity was seen with Musoke GP antisera. This difference correlated with the percentage of hybridoma clones producing infectivity-enhancing mAbs. Using mAbs to MARV GP, we identified 3 distinct ADE epitopes in the mucinlike region on Angola GP. Interestingly, some of these antibodies bound to both Angola and Musoke GPs but showed significantly higher ADE activity for strain Angola. ADE activity depended on epitopes in the mucinlike region and glycine at amino acid position 547, present in the Angola but absent in the Musoke GP.

Conclusions. These results suggest a possible link between ADE and MARV pathogenicity and provide new insights into the mechanisms underlying ADE entry of filoviruses.

Marburg virus (MARV) and Ebola virus (EBOV) are filamentous, enveloped, negative-strand RNA viruses belonging to the family Filoviridae. These viruses have produced sporadic outbreaks of hemorrhagic fever in human and nonhuman primates [1]. MARV was first identified in 1967 during an outbreak of hemorrhagic fever in Marburg, Germany, and Belgrade, Yugoslavia. These outbreaks were linked to infected monkeys imported from Uganda [2]. Since the first outbreak of Marburg hemorrhagic fever, several sporadic outbreaks have been reported in central African countries [1, 3–6]. The largest outbreak occurred in Uige province in Angola from 2004 to 2005, with a mortality rate of 90% among 252 reported cases [7]. The strain Angola had a higher mortality rate and was thought to be more pathogenic than earlier isolates, such as strain Musoke [8, 9], which was isolated from a human case in 1980 in Kenya [5]. Among EBOV species, *Zaire ebolavirus* seems to be the most virulent, with a case fatality rate of up to 90%; whereas *Reston ebolavirus* has never been associated with symptomatic infection in humans [10] and was shown to be less pathogenic than *Zaire ebolavirus* in nonhuman primates [11].

The filovirus genome encodes at least 7 structural proteins. The fourth gene from the 3' end of the genome encodes the envelope glycoprotein (GP), which undergoes proteolytic cleavage into 2 subunits, GP1 and GP2. The GP1 subunit mediates cell-surface receptor binding [12, 13], and the GP2 subunit is involved in fusion of the viral envelope and host cell membrane [14]. GP is highly glycosylated, with a large amount of N- and O-linked

Potential conflicts of interest: none reported.

Presented in part: 57th Annual Meeting of the Japanese Society for Virology, Tokyo, Japan, 25–27 October 2009.

Correspondence: Ayato Takada, PhD, Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Kita-20, Nishi-10, Kita-ku, Sapporo 001-0020, Japan (atakada@czc.hokudai.ac.jp).

The Journal of Infectious Diseases 2011;204:S978–S985

0022-1899 (print)/1537-6613 (online)/2011/204S3-0033$14.00
DOI: 10.1093/infdis/jir334

glycans, most of which are located in the middle portion of the GP, designated the mucinlike region (MLR) [15, 16]. The amino acid sequences of the MLR are highly variable among filovirus species [17, 18].

It has been demonstrated elsewhere that EBOV infection in humans and nonhuman primates induced GP-specific antibodies that had the ability to enhance viral infectivity of certain cells in vitro [19, 20]. This mechanism, known as antibody-dependent enhancement (ADE) of viral infection, depends mostly on the cross-linking of virus-antibody complexes through interaction with cellular Fc receptors (FcRs) [21]. Our previous studies have identified an additional mechanism underlying EBOV ADE in vitro, namely, complement protein C1q and C1q receptor-dependent ADE [19]. Epitopes involved in ADE were identified predominantly in the MLR of the Zaire EBOV (ZEBOV) GP1 subunit. A possible contribution of ADE to the distinct pathogenicity observed for ZEBOV and Reston EBOV was discussed elsewhere [20, 22]. However, little is known about the role of ADE in MARV pathogenicity.

In this study, we produced mouse antisera and monoclonal antibodies (mAbs) specific to the GPs of MARV strains Angola and Musoke and examined their ADE activities by using vesicular stomatitis virus (VSV) pseudotyped with MARV GPs. We found distinct ADE activity between antibodies to Angola and Musoke GPs, which may be linked to a difference in pathogenicity of these strains. The identified ADE epitopes were all located in the MLR of the GPs, but the presence of these epitopes was not sufficient to give a maximal ADE. Potential mechanisms for effective ADE seen with certain MARV strains are discussed here.

MATERIALS AND METHODS

Viruses and Cells

VSV pseudotyped with Angola GP (VSV-Angola) or Musoke GP (VSV-Musoke) expressing green fluorescent protein was generated as described elsewhere [23]. Deletion mutant GPs, chimeric GPs, and mutant GPs with a single substitution were generated as described elsewhere [24]. There was no significant difference in the infectivity in Vero E6 cells among these viruses, suggesting that the functional GPs were incorporated into VSV virions [24]. To reduce the background infectivity of parent VSV G, the pseudotyped viruses were treated with a neutralizing mAb to VSV G protein (VSV-G[N]1-9) before use. The virus infectivity was determined by counting the number of cells expressing green fluorescent protein, using fluorescence microscopy or flow cytometry. Monkey kidney Vero E6 cells and human embryonic kidney (HEK) 293 and 293T cells were grown in Dulbecco's modified Eagle's medium (Sigma), and human chronic myelogenous leukemia K562 cells bearing FcR were grown in Roswell Park Memorial Institute 1640 medium (Sigma). The media were supplemented with fetal calf serum and antibiotics.

Antisera

To produce antisera to filovirus GPs, 5-week-old female BALB/c mice were immunized subcutaneously twice in a 3-week interval with 100 μg of viruslike particles (VLPs) [25, 26] with complete Freund's adjuvant or intraperitoneally twice in a 3-week interval with 50 μg of VLPs only. The serum samples from intraperitoneally immunized mice were collected 7 days after the second immunization. Subcutaneously immunized mice were boosted intraperitoneally with 100 μg of VLPs alone 3 weeks after the second immunization, and the serum samples were collected 7 days after the boost dose.

Generation of mAbs

Five-week-old female BALB/c mice were immunized subcutaneously with 100 μg of VLPs with complete Freund's adjuvant (Difco). At 3 and 6 weeks after the first immunization, the mice were subcutaneously immunized with 100 μg of VLPs with incomplete Freund's adjuvant (Difco). Three weeks after the last immunization, mice were boosted intraperitoneally twice in a 3-week interval with 100 μg of VLPs only. Three days later, mouse spleen cells and mouse myeloma P3-U1 cells were fused and maintained according to a standard procedure [27]. Hybridomas were screened for secretion of MARV GP-specific mAbs by enzyme-linked immunosorbent assay (ELISA), and hybridoma-producing mAbs were cloned by limiting dilution of the cells. mAbs were purified from mouse ascites using protein A agarose columns (Bio-Rad). The isotypes of the obtained mAbs were determined using a mouse mAb isotyping test kit (AbD Serotec) according to the manufacturer's instructions.

Infectivity Enhancement and Neutralization Tests

Appropriately diluted serum samples or mAbs were mixed with equal volumes of the pseudotyped viruses ($\sim 10^5$ infectious units/mL on Vero E6 cells), followed by 1-hour incubation. Infectivity was then determined in Vero E6, K562, and HEK 293 cells for neutralizing, FcR-, and C1q-dependent ADE activities, respectively, by counting the fluorescent cells, as described elsewhere [19, 20, 22, 23]. The relative percentage of infected cells was determined by setting the number of cells infected in the absence of GP-specific antisera or purified mAbs to 100. Antibodies that gave relative infectivity values of $<50\%$ or $>200\%$ were defined as neutralizing or enhancing antibodies, respectively. For detection of C1q-dependent ADE, viruses were incubated with antisera or mAbs in the presence of C1q (50 μg/mL; Sigma) before infection of HEK 293 cells.

Enzyme-Linked Immunosorbent Assay

ELISA plates (Nunc Maxisorp) were coated with lysate from HEK 293T cells expressing MARV-GP, VLP, or histidine-tagged purified GP, as described elsewhere [28], at 4°C overnight and then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) before addition of blocking buffer (3% skim milk in PBST) for 2 hours at room temperature. After

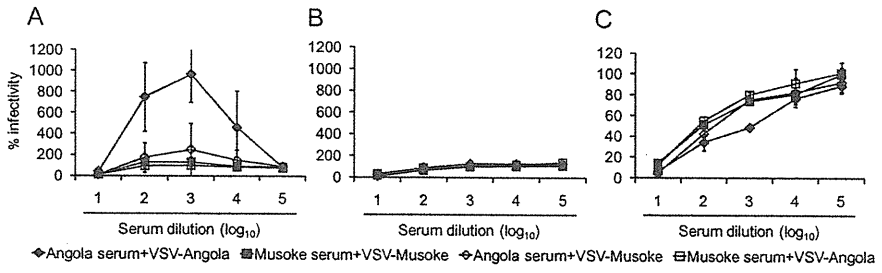

Figure 1. Antibody-dependent enhancement and neutralizing activities of polyclonal antisera from subcutaneously immunized mice. Marburg virus glycoprotein (MARV GP) antisera ($1:10^1$ to $1:10^5$ dilutions) were mixed with vesicular stomatitis virus (VSV) pseudotyped with MARV GPs, incubated for 1 hour at room temperature, and inoculated into 10^5 K562 cells (in 96-well plate) *A*, confluent HEK 293 cells *B*, or confluent Vero E6 cells *C*, Human embryonic kidney 293 cells were infected in the presence of purified C1q (50 μg/mL). Results are expressed as means (± standard deviations) of data for 3 immunized mice. Relative percentages of infected cells are shown as mean values determined based on the number of infected cells in the absence of specific antibodies against MARV GPs (100%).

being washed 3 times with PBST, primary antibodies (ie, hybridoma supernatants, purified mAbs, or antiserum) were added to each well and incubated at room temperature for 1 hour and the plates were washed 3 times with PBST. The antibody binding was detected by goat anti-mouse immunoglobulin (Ig) G1, IgG2a, IgG2b, IgG3 (Bethyl), and IgG (H+L) (Jackson ImmunoResearch) conjugated with horseradish peroxidase. After incubation at room temperature for 1 hour, the plates were washed 4 times with PBST, and 3,3',5,5'-tetramethylbenzidine (Sigma) was added to each well. An equal volume of 1N sulfuric acid was subsequently used to stop the enzyme reaction after 15-minutes incubation, and the optical density value was read at 450 nm on an ELISA plate reader.

Immunostaining

HEK 293T cells were transfected with the mammalian expression plasmid pCAGGS, expressing MARV GPs, and were fixed 48 hours later with methanol for 30 minutes. After blocking with 10% bovine serum albumin in PBS for 90 minutes at room temperature, cells were incubated with mAbs (1 μg/mL) for 1 hour at room temperature. The binding of the mAbs was detected by goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch) diluted in 5% bovine serum albumin in PBST. After incubation for 1 hour at room temperature, GP expressed in the cells was visualized with 3,3'-diaminobenzidine.

RESULTS

FcR-Dependent ADE Activity of GP Antisera Differed Between MARV Strains Angola and Musoke

To examine ADE of MARV infection, we first generated antiserum by subcutaneous immunization of mice with MARV strain Angola or Musoke VLPs. The levels of infectivity of VSV-Angola and VSV-Musoke in the presence of these antisera were determined by using K562 and HEK 293 cells for FcR- and C1q-dependent ADE, respectively (Figure 1*A* and 1*B*). In K562 cells, the infectivity of VSV-Angola was markedly enhanced in the presence of Angola GP antiserum at dilutions ranging from $1:10^2$ to $1:10^4$, whereas only minimal enhancement of VSV-Musoke was seen in the presence of Angola GP antiserum. In the presence of Musoke GP antiserum, no apparent enhancement of infectivity was observed for any of the 2 VSV pseudotypes. Furthermore, no C1q-dependent ADE activity in HEK 293 cells was detected in the Angola or Musoke GP antiserum at any dilution (Figure 1*B*), and no significant difference in neutralizing activity was observed between the 2 antisera (Figure 1*C*). Interestingly, cross-reactivity between Angola and Musoke GP antiserum was found in their neutralizing activities but not in FcR-dependent ADE activities.

Difference in C1q-Dependent ADE Activity Between MARV and EBOV Antisera

It was demonstrated elsewhere that intraperitoneal immunization elicited C1q-dependent ADE antibodies to ZEBOV GP more efficiently than subcutaneous immunization [20]. Therefore, we produced MARV strain Angola and Musoke GP antisera by intraperitoneal immunization and compared C1q-dependent ADE activity with ZEBOV GP antisera in HEK 293 cells (Figure 2*A*). We confirmed that ZEBOV GP antisera significantly enhanced the infectivity of VSV pseudotyped with ZEBOV GP in the presence of purified C1q. However, the Angola GP antiserum only minimally enhanced the infectivity of VSV-Angola in the presence of C1q. The Musoke GP antiserum did not show any C1q-dependent ADE activity. Subsequently, we used ELISA to examine the antiserum for the proportion of each IgG subclass (Figure 2*B*). We found that the amount of IgG2a, IgG2b, and IgG3 was significantly lower in the MARV-Angola GP antiserum than in the ZEBOV GP antisera (Student's *t* test, $P < .05$). There was no significant difference in the amount of IgG1 between the MARV-Angola GP and ZEBOV GP antisera.