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 Ladislav 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 Chulu7 and Ayato Takada1,2

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

1Hokkaido University Research Center for Zoonosis Control, Kita-20, Nishi-10, Kita-ku, Sapporo 001-0020, Japan
2Hokudai Center for Zoonosis Control in Zambia, School of Veterinary Medicine, The University of Zambia, PO Box 32379, Lusaka, Zambia
3Department of Disease Control, School of Veterinary Medicine, The University of Zambia, PO Box 32379, Lusaka, Zambia
4Department of Paraclinical Studies, School of Veterinary Medicine, The University of Zambia, PO Box 32379, Lusaka, Zambia
5Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
6Japan Science and Technology Agency Basic Research Programs, Saitama, Japan
7Zambia 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.

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
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 over-wintering 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 conform to that described previously by Bahl et al. (2009), particularly in the classification of isolates into the American and
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. 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.

Phylogenetic analysis of 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).

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

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

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

*The first influenza virus isolate from an avian host in Zambia (Simulundu et al., 2009).
viruses grouped with those of AIVs isolated from wild birds in Zambia. 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. 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 44–1086 (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.
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. 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.
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.
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 found 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).

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

<table>
<thead>
<tr>
<th>Protein</th>
<th>Aa position</th>
<th>Host</th>
<th>Isolate†</th>
<th>Avian</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>475</td>
<td>L</td>
<td>M</td>
<td>Zb08 (H6N2)</td>
<td>Zb10 (H6N2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zb07 (H6N2)</td>
<td>Zb13 (H9N1)</td>
</tr>
<tr>
<td>PB1-F2</td>
<td>66</td>
<td>N</td>
<td>S†</td>
<td>Zb13 (H9N1)</td>
<td>Zb13 (H9N1)</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>V</td>
<td>A</td>
<td>Zb13 (H9N1)</td>
<td>Zb13 (H9N1)</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>L</td>
<td>S</td>
<td>Zb04 (H3N8)</td>
<td>Zb05 (H3N8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zb06 (H3N8)</td>
<td>Zb06 (H3N8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zb08 (H6N2)</td>
<td>Zb10 (H6N2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zb10 (H6N2)</td>
<td>Zb13 (H9N1)</td>
</tr>
<tr>
<td>M2</td>
<td>28</td>
<td>I</td>
<td>V</td>
<td>Zb04 (H3N8)</td>
<td>Zb08 (H6N2)</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>L</td>
<td>F</td>
<td>Zb07 (H4N6)</td>
<td>Zb08 (H6N2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zb08 (H6N2)</td>
<td>Zb10 (H6N2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zb10 (H6N2)</td>
<td>Zb12 (H11N9)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. positive/ total</th>
<th>Mean virus titre of positive samples (log_{10} EID_{50} g^{-1})</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung</td>
<td>Brain</td>
<td></td>
</tr>
<tr>
<td>Zb03 (H6N2)</td>
<td>3/5</td>
<td>3.3 &lt;10^{1.5}</td>
<td>–</td>
</tr>
<tr>
<td>Zb04 (H3N8)</td>
<td>5/5</td>
<td>3.6 &lt;10^{1.5}</td>
<td>0.099</td>
</tr>
<tr>
<td>Zb10 (H6N2)</td>
<td>5/5</td>
<td>4.8 &lt;10^{1.5}</td>
<td>0.001‡</td>
</tr>
</tbody>
</table>

*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.

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 HKY (plus invariant sites) model, the GTR+I (plus invariant sites) model, and the GTR+I+G model 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 anesthetized 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 clarified by centrifugation and titrated in 10–11-day-old embryonated chicken’s eggs. The tissue 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


Characterization of avian influenza viruses in Zambia


