The zoonotic potential of avian influenza viruses isolated from wild waterfowl in Zambia

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Abstract

Whilst remarkable progress in elucidating the mechanisms governing interspecies transmission and pathogenicity of highly pathogenic avian influenza viruses (AIVs) have been made, similar studies focusing on low pathogenic AIVs isolated from the wild waterfowl reservoir are limited. We previously reported that two AIV strains (subtypes H6N2 and H3N8) isolated from wild waterfowl in Zambia harbored some amino acid residues preferentially associated with human influenza virus proteins (so-called human signatures) and replicated better in the lungs of infected mice and caused more morbidity than a strain lacking such residues. To further substantiate these observations, we infected chickens and mice intranasally with AIV strains of various subtypes (H3N6, H3N8, H4N6, H6N2, H9N1 and H11N9) isolated from wild waterfowl in Zambia. Although some strains induced seroconversion, all the tested strains replicated poorly and were nonpathogenic for chickens. In contrast, most of the strains having human signatures replicated well in the lungs of mice, and one of these strains caused severe illness in mice and induced lung injury that was characterized by a severe accumulation of polymorphonuclear leukocytes. These results suggest that some strains tested in this study may have the potential to directly infect mammalian hosts without adaptation, which might possibly be associated with the possession of human signature residues. Close monitoring and evaluation of host-associated signatures may help elucidate the prevalence and emergence of AIVs with potential of causing zoonotic infections.

Introduction

It is widely acknowledged that avian influenza viruses (AIVs) circulating in their wild waterfowl reservoir such as ducks, gulls and shorebirds occasionally transmit to land-based avian hosts and mammals [24, 33]. Most AIVs found in the reservoir generally cause asymptomatic or less severe diseases in poultry and are thus referred to as low pathogenic AIVs (LPAIVs). In contrast, highly pathogenic AIVs (HPAIVs) cause an acute systemic disease in poultry with a mortality rate that often approaches 100% [1]. Hitherto, only some AIVs of the H5 and H7 subtypes are known to become HPAIVs under natural conditions. It is also known that AIVs of limited subtypes (H1-H3) have so far established stable lineages in some non-avian species such as humans, pigs and horses [25, 33].

In recent years, repeated zoonotic transmissions of AIVs (mostly H5, H7 and H9 subtypes) from terrestrial birds into humans have occurred [7, 25]. In fact, prior to the emergence of the 2009 H1N1 pandemic virus, the unprecedented impact on animal and public health of the Asian origin H5N1 HPAIV led to the prediction that a virus of subtype H5N1 might cause the next pandemic [21]. These zoonotic transmissions have been influential in heightening investigations into the virulence and host range determinants of AIVs, particularly those for HPAIVs [2, 21]. Whereas remarkable progress has been made in understanding the pathogenesis of HPAIVs, information on virulence and host range determinants of LPAIVs isolated from the waterfowl reservoir is minimal. This is despite the fact that the past documented pandemics (i.e. 1918 H1N1, 1957 H2N2, 1968 H3N2 and 2009 H1N1) were caused by viruses of non-highly pathogenic subtypes

and possessed some genes of avian origin. Moreover, the recent pandemic threat due to a novel reassortant avian-origin influenza A (H7N9) virus found in China is of low pathogenic pathotype [12]. Furthermore, there is mounting evidence suggesting that LPAIVs of multiple subtypes can infect mammals including humans under natural and experimental conditions, with appreciable degrees of morbidity and mortality [7, 9, 14, 17, 20, 22, 25, 30]. In the context of influenza pandemic preparedness, these data indicate that prudent pandemic plans should involve research and surveillance efforts targeting most influenza virus subtypes worldwide.

To date, the key amino acid substitutions that may enable an AIV to cause interspecies transmissions into other hosts remain obscure. By analyzing a large data set of influenza virus sequences, several studies have identified host-specific, conserved amino acids at particular positions, so-called genetic signatures [5, 6, 10, 27]. It has been hypothesized that these specific substitutions might be associated with the ability of AIVs to efficiently infect mammalian hosts including humans. However, this notion has not been comprehensively tested. We previously reported that some AIV strains isolated from wild waterfowl in Zambia harbored residues frequently observed in human influenza viruses [28]. In experimental infection of mice, two AIV strains possessing the human signature residues showed higher levels of virus replication in the lungs of infected mice and caused more morbidity as measured by weight loss than a strain lacking such residues. To further substantiate these observations, we assessed the replicative and pathogenic potential of several Zambian AIV strains (subtypes H3N6, H3N8, H4N6, H6N2, H9N1 and H11N9) in chicken and mouse models.

Materials and methods

Ethics statement

All animal experimental procedures were conducted in the biosafety level 2 and 3 facilities at Hokkaido University Research Center for Zoonosis Control, Japan, in strict accordance to the guidelines of the institutional animal care and use committee of Hokkaido University.

Viruses

AIVs used in this study (Table 1) were isolated from wild waterfowl in Zambia between 2006 and 2009 [28, 29]. Before being used in this study, these viruses were passaged once in 10-day-old specific-pathogen-free (SPF) embryonated eggs and then titrated to determine the EID_{50} calculated by a method described previously [26]. The viruses were appropriately diluted with sterile phosphate-buffered saline (PBS) to adjust the virus titer to $10^{7.5}$ EID_{50}/ml.

Experimental infection of chickens

Viruses were inoculated intranasally (i.n.) into 6-week-old SPF chickens (Boris brown) at $10^{7.5}$ EID_{50}/ml (0.1 ml). There were nine groups (including a PBS-inoculated control group) in total, consisting of six chickens each. On day 3 post-inoculation (p.i.), three chickens were euthanized and tracheas, lungs, and colons were sampled for virus titration in eggs. The rest of the chickens were monitored for clinical signs for 14 days. At the end of this period, serum was obtained for antibody titration by a standard haemagglutination inhibition (HI) test.

Experimental infection of mice

The methods used to investigate the replicative and pathogenic capacity of these viruses in mice were essentially the same as those previously described [28], with slight modifications. Here, 6-week-old female BALB/c mice (15 mice per group) were inoculated i.n. with 0.05 ml of Zb01

(H3N6), Zb05 (H3N8), Zb07 (H4N6), Zb13 (H9N1), Zb11 (H11N9) or Zb12 (H11N9) ($10^{7.5}$ EID_{50}/ml). Mock-infected mice received the same volume of PBS. Five mice were euthanized on days 1 and 3 p.i. for virus titration in the lungs and spleens. Monitoring of mice for clinical signs and virus titration in organs were done as described previously [28].

Histopathology and immunohistochemistry

BALB/c mice (three mice per group) were infected i.n. with 0.05 ml of $10^{7.5}$ EID_{50}/ml of Zb01 (H3N6) and Zb07 (H4N6), or mock-infected with sterile PBS. On day 3 p.i., mice were euthanized and whole lung tissues were collected and fixed with buffered neutral formalin and then paraffin-embedded. Formalin-fixed paraffin-embedded tissues were cut into sections of 3 μm thickness and mounted on glass slides for histopathological and immunohistochemical examination. For histopathological assessment, the sections were stained with hematoxylin and eosin or only hematoxilin and microscopically examined. For immunohistochemical staining, we used anti-influenza A virus nucleoprotein (NP) rabbit polyclonal hyperimmune serum (1:5000; rabbit was immunized with amino acids 428-441 of NP) for detection of influenza viruses. For immunohistochemical staining of viral NP antigens retrieval was performed with a pressure cooker using 0.01 M citrate buffer (pH 6.0). After cooling and washing in PBS, endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol for 15 minutes at room temperature (RT). The sections were then washed in PBS and incubated for 10 minutes with normal goat serum (Nichirei, Tokyo, Japan) at RT and for 30 minutes with primary antibody (anti-NP rabbit antiserum) at RT. The sections were then incubated for 30 min at RT with a secondary antibody labeled with horseradish peroxidase (Histofine Simple Stain MAX-PO(R); Nichirei). Immunoreactivity was detected using a 3, 3'-diaminobenzidine substrate.

Results

Waterfowl LPAIVs replicated poorly and caused no disease in chickens

To ascertain the ability of the LPAIV strains isolated from waterfowl in Zambia to replicate in terrestrial birds, chickens were inoculated i.n. with each of the viruses listed in Table 1. Among the viruses tested in chickens, Zb01 (H3N6), Zb03 (H6N2) and Zb11 (H11N9) apparently possessed no human signature residues, whilst the rest of the viruses had at least one such residue (Table 2). During the observation period, none of the chickens showed any symptoms of illness. Virus was not detected in any of the trachea, lung and colon samples collected on day 3 p.i., suggesting their poor replicative capacity in this animal model. However, at least one of the three chickens inoculated with Zb01 (H3N6), Zb07 (H4N6), Zb10 (H6N2), Zb11 (H11N9) and Zb12 (H11N9) produced virus-specific antibodies as indicated by increased HI titers that ranged from 16 to 128 (Table 1). These results suggest that the seroconverted chickens had been infected despite no recovery of inoculated viruses from their tissue samples. With regards to the replicative and/or pathogenic potential of the viruses tested, we did not discern any appreciable differences between viruses possessing and lacking human signatures.

Waterfowl LPAIVs possessing human signatures replicated well in lungs of infected mice

Next, we investigated the ability of the LPAIV strains to replicate in mouse lungs. Since the replicative and pathogenic profiles of Zb03 (H6N2) and Zb10 (H6N2) were determined previously (i.e., Zb10 (H6N2), possessing 4 human signature residues, replicated better and caused more severe disease in mice than Zb03 (H6N2) which lacks such residues) [28], these viruses were excluded in this mouse experiment. On day 1 p.i., all the viruses tested were

recovered from the lungs of all infected mice with virus titers ranging from $10^{3.5}$ to $10^{5.3}$ EID_{50}/g (Table 3). Marked differences in lung virus titers among the viruses were observed on day 3 p.i.; Zb01 (H3N6), Zb11 (H11N9) and Zb12 (H11N9) displayed poor replication (virus titers were ≤ $10^{1.8}$ EID_{50}/g), whereas Zb05 (H3N8), Zb07 (H4N6) and Zb13 (H9N1) exhibited higher levels of virus replication with virus titers ranging from $10^{2.7}$ to $10^{3.9}$ EID_{50}/g (Table 3). It was also noted that viruses were detected in the lungs of all the mice inoculated with Zb05 (H3N8), Zb07 (H4N6) and Zb13 (H9N1), whereas in Zb01 (H3N6), Zb11 (H11N9) and Zb12 (H11N9)-inoculated mice, virus was detected only in one, two and three of the 5 mice, respectively (Table 3). None of the viruses was detected in the spleen, suggesting that the viruses could not efficiently spread systemically. An apparent correlation between possession of human signatures and capacity to efficiently replicate in the lungs of mice was implicated by Zb05 (H3N8), Zb07 (H4N6) and Zb13 (H9N1)-inoculated mice. However, whilst having a human signature residue, Zb12 (H11N9) replicated poorly in the lungs of infected mice.

Waterfowl LPAIVs induced illness in mice

We then investigated the capacity of Zb01 (H3N6), Zb05 (H3N8), Zb07 (H4N6), Zb13 (H9N1), Zb11 (H11N9) and Zb12 (H11N9) to cause illness in mice. Mice were monitored for clinical signs such as inappetence, labored breathing, weight loss, ruffled fur and hunching for 14 days. Except for Zb07 (H4N6)-infected mice which exhibited severe weight loss, a transient weight loss of up to about 11% was observed between days 1 and 4 in infected mice, and their weight returned to baseline by day 8 p.i. (Fig. 1). It was noted that a remarkable reduction of body weight (more than 10%) was seen in Zb05 (H3N8) - and Zb07 (H4N6)-infected mice. Moderate ruffled fur was seen in mice infected with Zb01 (H3N6), Zb05 (H3N8) and Zb13 (H9N1). Zb07

(H4N6)-infected mice showed severe ruffled fur, labored breathing and hunching for the most part of the observation period. All the mice survived the infection for the 14-day observation period. It was noteworthy that in an independent experiment in which undiluted chorioallantoic fluid was used, mice infected with Zb07 (H4N6) (10^9 EID_{50}/ml/mouse) died within 5 days p.i., whilst all mice infected with other viruses survived the infection (data not shown).

Zb07 (H4N6)-infected mice exhibited severe pulmonary lesions

To examine the lung pathology induced by Zb07 (H4N6), mice were infected i.n. with Zb01 (H3N6) and Zb07 (H4N6). Zb01 (H3N6) was selected for histopathological analyses because it induced the most weight loss among the strains lacking human signatures. On day 3 p.i., a time point at which most isolates from Zambia caused maximal weight loss, mice were euthanized and whole lung tissues were collected and processed for histopathological examination. Lungs of mice infected with Zb01 (H3N6) showed moderate interstitial and perivascular infiltration of inflammatory cells as well as desquamation of the bronchial epithelium and migration of macrophages to alveolar space (Fig. 2c and d). On the other hand, lung tissues of mice infected with Zb07 (H4N6) exhibited severe infiltration of inflammatory cells into the perivascular and interstitial space (Fig. 2e and f). Interstitial edema and migration of inflammatory cells to alveolar space were also observed in lung tissues of Zb07 (H4N6)-infected mice (Fig. 2f). Though perivascular infiltration of inflammatory cells was observed in lungs of mice infected with Zb01 (H3N6) and Zb07 (H4N6), interestingly, the inflammatory cellular compositions in these areas were different. We observed that infiltration of polymorphonuclear leucocytes (PMNs), mostly neutrophils, in the lung tissues of Zb07 (H4N6)-infected mice was more prominent than in those of Zb01 (H3N6)-infected mice. To confirm this observation, we

prepared sections stained with only hematoxylin and morphologically analyzed the inflammatory cellular compositions of perivascular areas (Fig. 3). We found that inflammatory cells were mainly lymphocytes in most of perivascular areas of the lungs of Zb01 (H3N6)-infected mice (Fig. 3a). On the other hand, PMNs were predominantly found in perivascular areas of the lung tissues of Zb07 (H4N6)-infected mice (Fig. 3b). By immunohistochemistry for the NP antigen, positive signals were observed in bronchial and alveolar epithelium cells of the lungs of both Zb01 (H3N6) and Zb07 (H4N6)-infected mice (Fig. 3c and d).

Discussion

Herein, we utilized animal models (chickens and mice) to biologically characterize LPAIV strains isolated from wild waterfowl in Zambia. In chickens, all the tested viruses replicated poorly, whilst five isolates induced seroconversion in at least one of the three virus-inoculated chickens (Table 1). This finding may be consistent with AIVs that are not adapted to gallinaceous poultry. However, in the context of avian influenza surveillance and control, the existence of wild waterfowl AIVs which can directly infect chickens, but causing no obvious signs of disease poses an epidemiological challenge in intensive monitoring of commercial or backyard poultry. Frequent introduction of such viruses into poultry may lead to an increased risk that AIVs acquire mutations that may allow them to expand their host range [15]. In fact, a serological study has recently provided evidence of human infections with LPAIVs (subtype H4 and H11) among backyard poultry growers [18]. As clearly demonstrated by the recent human cases of H7N9 LPAIV infection in China, contact with unrecognized LPAIV-infected domestic birds may indeed lead to human infections with dire consequences.

In stark contrast to the findings in chicken experiments in which there were no appreciable differences between viruses possessing and lacking human signature residues, most LPAIV strains having human signatures exhibited better replication in mice than those lacking such residues (Table 3). Importantly, all the tested viruses induced illness in mice. With the exception of Zb07 (H4N6) which caused the most severe disease in mice, there were no marked differences in the severity of disease between mice infected with viruses either possessing or lacking human signatures. Taken together, these data suggest that even wild waterfowl AIVs displaying poor

replicative capacity in gallinaceous poultry may have the potential to directly switch hosts and infect mammals without prior adaptation. Although most reported human infections with AIVs occurred as a result of close contact with domestic poultry, serologic evidence of AIV infection (subtype H11N9) in three individuals with a history of substantial exposure to wild waterfowl and game birds does support the idea of direct transmission of AIVs from their natural reservoirs into humans [13].

Whilst it might be reasonable to assume that the presence of human signature residues in some of the waterfowl LPAIV strains tested may have influenced the observed improved replication in mice, further studies employing techniques such as reverse genetics and site-directed mutagenesis are required to reach such a conclusion. Moreover, despite having a human signature residue, Zb12 (H11N9) replicated poorly in the lungs of infected mice. Even as this finding may indicate that the enhanced replication of LPAIVs in mice observed in the current study may not solely be accounted for by possession of human signature residues, it may also imply that some of these residues may not be critical factors in host-switch mechanisms of AIVs. Other viral (e.g. subtype; H11 viruses are rarely detected in non-reservoir hosts) and host factors may be involved. Regardless, our studies provide impetus to investigate how AIVs may acquire human/mammalian associated residues in nature and the potential role of such residues in virulence and/or host-switch mechanisms of AIVs.

Histopathological examination revealed that wild bird LPAIVs, Zb01 (H3N6) and Zb07 (H4N6), induced moderate and severe pulmonary lesions in mice, respectively, a result which appeared to correlate with the magnitude of virus replication and morbidity caused by these viruses (Table 3

and Fig. 1). The finding that lung lesions of Zb07 (H4N6)-infected mice were severer than those of mice infected with Zb01 (H3N6) may also be related to the greater early inflammatory response characterized by a severe accumulation of PMNs observed in the perivascular lesions of Zb07 (H4N6)-infected mice than those of Zb01 (H3N6) (Fig. 3). Whereas these cells have been shown to play a significant role in preventing influenza virus propagation in the lungs following primary pulmonary infection, excessive influx of PMNs, particularly neutrophils may contribute to acute lung injury in influenza virus-induced pneumonia [11, 23, 31]. It is unclear why Zb07 (H4N6) induced a greater influx of PMNs into mouse lung than Zb01 (H3N6). Although the level of virus replication may be involved, the expression of inflammatory mediators such as complement factors and chemokines may also play roles in inducing PMNs migration into the mouse lung [32]. It is also worth noting that Zb07 (H4N6) possesses human associated residues in the matrix 2 (M2) protein. The M2 protein has been shown to induce secretion of the pyrogenic cytokine IL-1β via stimulation of the NLRP3 inflammasome pathway [16]. This may contribute to uncontrolled deleterious inflammation (so-called cytokine storm) which may exacerbate lung immunopathology and disease of influenza [3]. Furthermore, IL-1 plays an important role in hemostasis deregulation through tissue factor induction. Since hemostasis deregulation emerges as a key pathway in cytokine storm induced by influenza viruses [3, 4, 18], it seems reasonable to speculate that human signature residues in the M2 protein of AIVs may be involved in cytokine storm via IL-1 production and hemostasis deregulation.

In this study, we have demonstrated that some of the LPAIVs isolated from wild waterfowl in Zambia may have the potential to infect chickens and mice without adaptation. We have also shown possible correlation between viral replication capacity in mice and possession of human

signature residues in viral proteins, which may be associated with the potential to cause interspecies transmissions. Our study emphasizes the need for close monitoring and evaluation of host-associated signatures in AIVs to better understand the emergence of strains capable of causing zoonotic infections.

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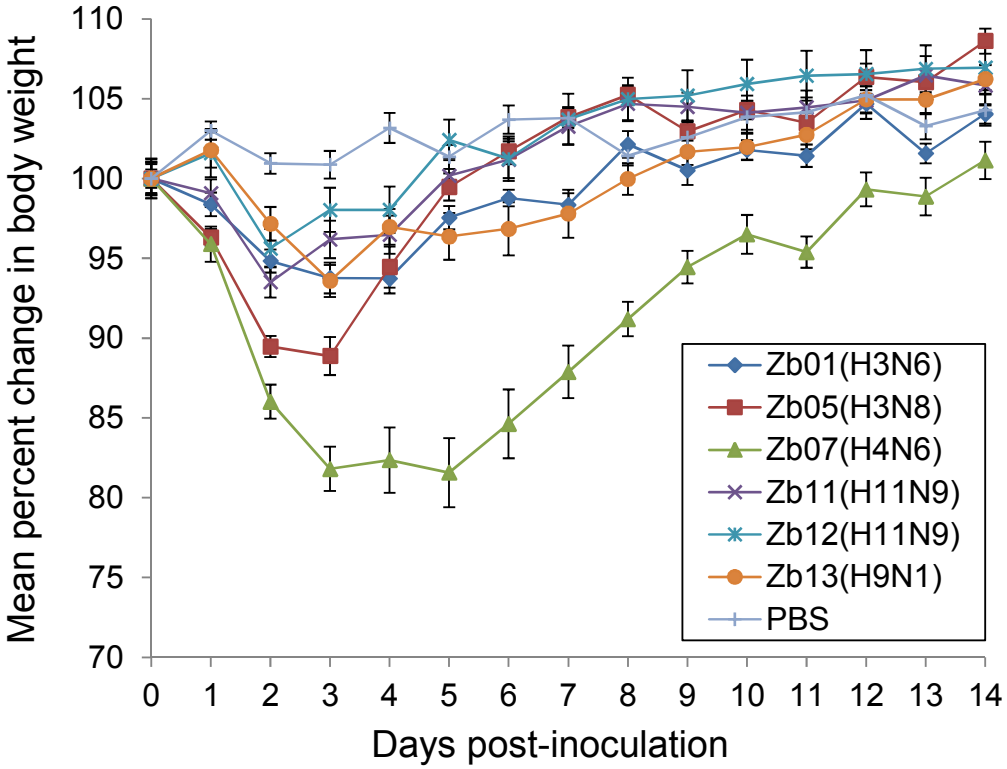
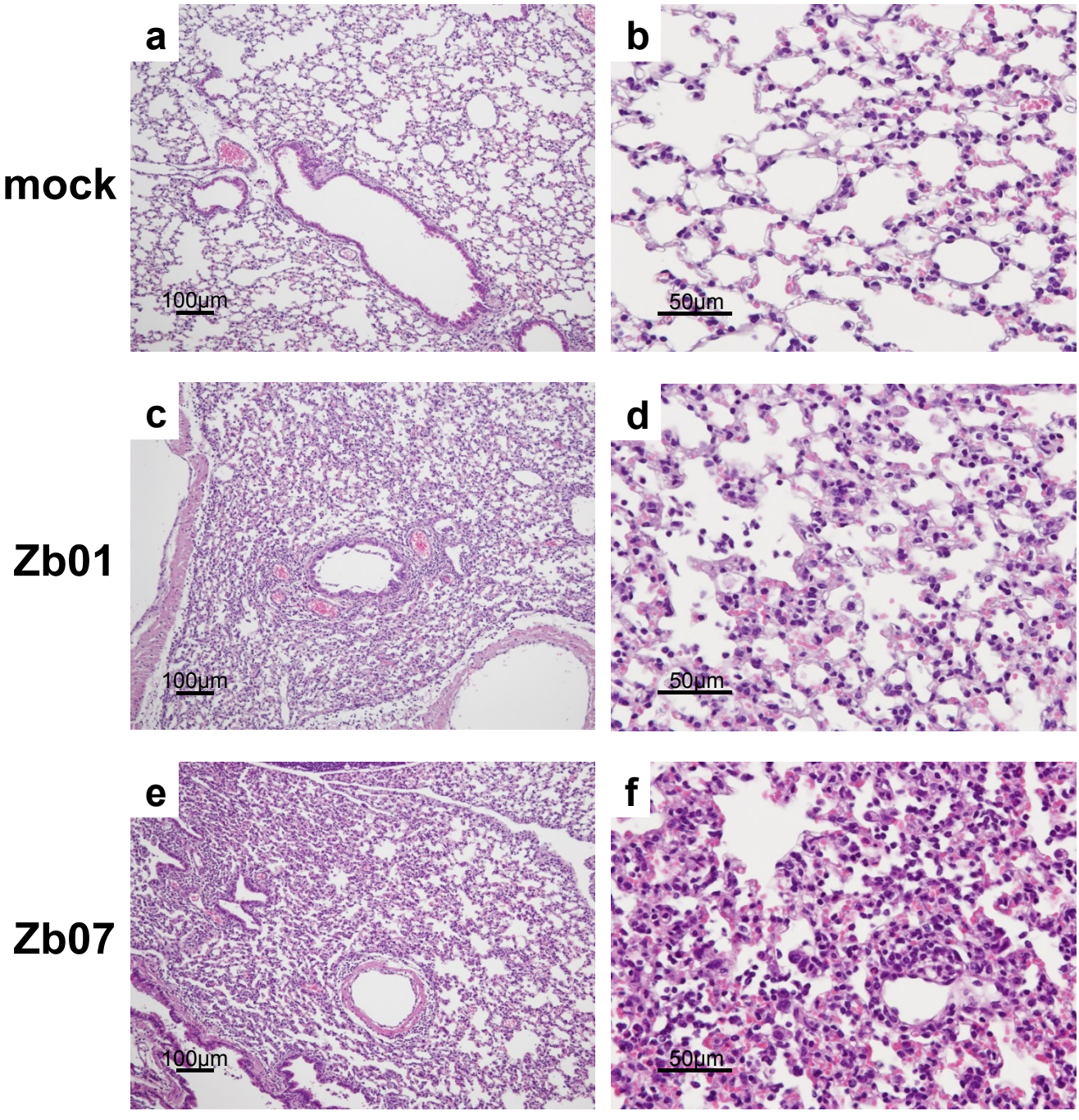
Figure legends

Fig. 1. Weight loss in mice infected with LPAIVs isolated from wild waterfowl in Zambia. Data are presented as mean body weight change per group ± standard deviation.

Fig. 2. Representative histopathological images of lung tissues of mice infected i.n. with Zb01 (H3N6) or Zb07 (H4N6). On day 3 p.i., lung samples were collected from mock399 infected (a and b), Zb01 (H3N6)-infected (c and d) and Zb07 (H4N6)-infected (e and f) mice. The tissue sections were stained with hematoxylin and eosin.

Fig. 3. Representative nuclear staining and immunohistochemical images of lung sections of mice infected i.n. with Zb01 (H3N6) or Zb07 (H4N6). On day 3 p.i., lung samples were collected from Zb01 (H3N6)-infected (a and c) and Zb07 (H4N6)-infected (b and d) mice. The tissue sections were stained with hematoxylin alone for nuclear stain (a and b), or rabbit polyclonal antibody against viral NP antigens for immunohistochemistry (c and d).


Mean percent change in body weight
110
105
100
95
90
85
80
75
70
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days post-inoculation
Zb01(H3N6)
Zb05(H3N8)
Zb07(H4N6)
Zb11(H11N9)
Zb12(H11N9)
Zb13(H9N1)
PBS


mock
Zb01
Zb07
a
b
c
d
e
f
100μm
50μm
100μm
50μm
100μm
50μm

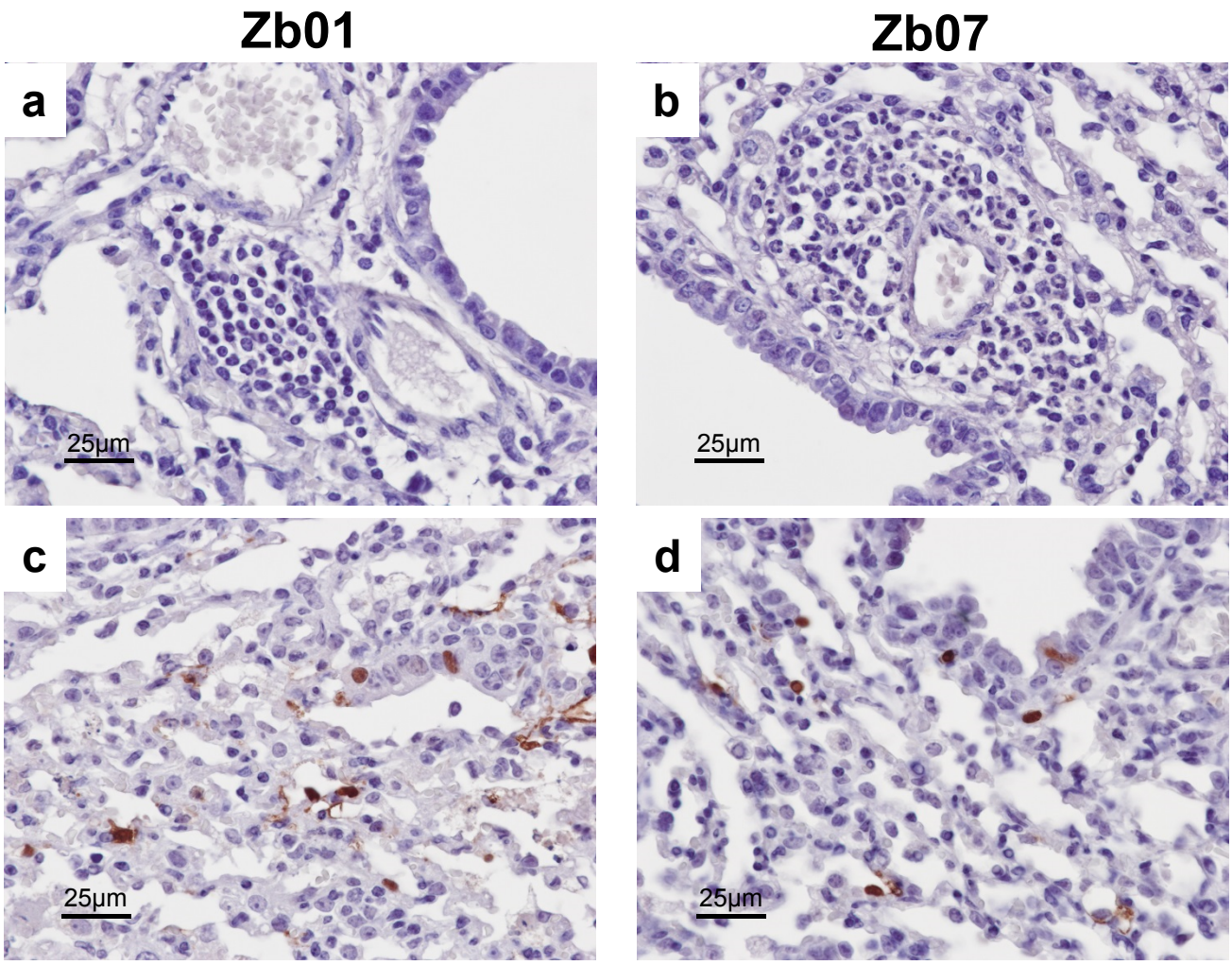

Zb01
Zb07
a
b
c
d
25μm
25μm
25μm
25μm

Table1

Table 1. Seroconversion of chickens inoculated with LPAIVs isolated from wild waterfowl[1]

Virus	Abbreviation	HI titers of three individual chickens 14 days after inoculation		
A/pelican/Zambia/01/06 (H3N6)	Zb01 (H3N6)[2]	16	8	16
A/goose/Zambia/05/08 (H3N8)	Zb05 (H3N8)[3]	<2	8	4
A/goose/Zambia/07/08 (H4N6)	Zb07 (H4N6)[3]	16	64	<2
A/duck/Zambia/03/08 (H6N2)	Zb03 (H6N2)[2]	<2	<2	<2
A/duck/Zambia/10/09 (H6N2)	Zb10 (H6N2)[3]	<2	128	64
A/pelican/Zambia/13/09 (H9N1)	Zb13 (H9N1)[3]	8	<2	<2
A/duck/Zambia/11/09 (H11N9)	Zb11 (H11N9)[2]	<2	32	<2
A/duck/Zambia/12/09 (H11N9)	Zb12 (H11N9)[2]	32	<2	<2
	PBS	<2	<2	<2

[1]Serum with HI titers ≥16 was considered positive.

[2]Virus with no apparent human/mammalian-associated residues in its proteins.

[3]Virus having at least one human/mammalian-associated residue in its proteins.

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

Protein	Position[1]	Host		Isolate[2]
		Avian	Human	
PB2	475	L	M	Zb10 (H6N2)
PB1-F2	66	N	S[3]	Zb13 (H9N1)
	76	V	A	Zb13(H9N1)
	82	L	S	Zb05 (H3N8)
				Zb10 (H6N2)
				Zb13 (H9N1)
	87	E	G	Zb10 (H6N2)
M2	55	L	F	Zb07 (H4N6)
				Zb10 (H6N2)
				Zb12 (H11N9)

[1]Human-associated residues at these specific positions were describe previously [5, 10, 27].

[2]Names of isolates possessing human-associated amino acid residues.

[3]The amino acid serine at position 66 of the PB1-F2 protein is not a human-associated residue, but was previously shown to increase virulence in mice [8].

Table 3. Replication of LPAIVs isolated from wild waterfowl in mice

Virus	Mean titers (log_{10} EID_{50}/g) of virus-positive samples		
	Lung		Spleen
	Day 1	Day 3	Day 3
Zb01 (H3N6)[1]	3.7 (5/5)	1.5 (1/5)	<1.5
Zb05 (H3N8)[2]	3.9 (5/5)	2.7 (5/5)	<1.5
Zb07 (H4N6)[2]	5.3 (5/5)	3.9 (5/5)	<1.5
Zb11 (H11N9)[1]	4.0 (5/5)	1.8 (2/5)	<1.5
Zb12 (H11N9)[2]	4.0 (5/5)	1.7 (3/5)	<1.5
Zb13 (H9N1)[2]	3.5 (5/5)	3.4 (5/5)	<1.5

[1]Virus with no apparent human/mammalian-associated residues in its proteins.

[2]Virus having at least one human/mammalian-associated residue in its proteins.