Characterization of an H6N1 avian influenza virus from an illegally imported product

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Abstract

Background : H6N1 is low pathogenic virus commonly found in wild and domestic birds across many continents. To investigate the infective status of imported food, we detected avian influenza virus (AIV) in illegally imported frozen food from international flight.

Methods : The virus was isolated, the complete genome was sequenced, and the pathogenicity was tested. PQIATR/G was found in the motif of the HA cleavage site of the isolated strain, which conforms to the molecular characteristics of a low pathogenic AIV. The amino acid residues at positions 186, 190, 226, and 228 of the HA protein receptor binding sites were proline (P), valine (V), glutamine (Q), serine (S), respectively, exhibiting the molecular characteristics required to bind to human-like receptors. The SPF chicken test showed that the IVPI was 0 and the virus was a low pathogenic AIV. The infectious results in BALB/c mice showed that the isolate could be replicated in the lower respiratory tract of mice. Mice infected with a high dose of the virus displayed moderate weight loss but no obvious clinical symptoms.

Results :The results of the sequence analysis showed that the avian influenza virus was of the H6N1 subtype, which shared the highest nucleotide identity with the AIV subtypes prevalent in Taiwan province.

Conclusions : It is important to intensily monitor the illegal carrying.

Background

Avian influenza viruses (AIVs) have caused huge economic losses to the global poultry industry and posed a great threat to global public health [1–2]. Currently, 16 HA subtypes (H1–H6) and 9 NA (N1–N9) of influenza viruses have been isolated from birds[3]. AIVs can be divided into highly pathogenic AIV (HPAIV) and low pathogenic AIV (LPAIV). HPAIV can directly infect waterfowl and poultry, and may occasionally infect humans. Moreover, LPAIV may lead to the generation of HPAIV through genetic mutations or assortment. Thus, LPAIV infections are becoming a major threat to the poultry industry and only limited protection is provided by the available inactivated vaccines due to an improper cold chain or administered dose [4]. Although migratory birds and commercial live poultry have been considered to be major factors associated with spread of HPAIVs, smuggled avian products are also risk factors for AIV spread internationally. In 2005, a low pathogenic H10N7 AIV was isolated from one of the smuggled duck carcasses, which was later seized by official controls in a warehouse in Italy[5]. From 2015 to 2016, highly (H5N1 and H5N6) and low (H9N2 and H1N2) pathogenic AIVs were isolated from raw chickens and duck products illegally imported to Japan by international passengers[6].

On Oct 13th, 2017, a frozen chicken illegally introduced into China by international passengers was seized. In this study, a low pathogenic H6N1 AIV was isolated from this chicken. The results clearly show that illegal raw products can contribute to the spread of AIV and we should intensely monitor such illegal animal transportation.

Methods

Poultry product confiscated at inspection and quarantine

One frozen uncooked chicken (Figure 1) was confiscated at Pudong International Airport by a passenger from airline CI501 flying from Taiwan province to Shanghai.

Viral RNA extraction

The liver and lung issues of the frozen chickens were collected and cut into small pieces and placed in a tube with magnetic beads followed by rubbing. The homogenate was then centrifuged at 10000 rpm for 10 min, and the supernatants were used for RT-PCR detection.

Real-time RT-PCR

The supernatants were detected using an influenza A RT-PCR assay LE (Qiagen, Hilden, Germany) for all known influenza viruses. A H5/H7/H9 subtype (Qiagen, Hilden, Germany) kit was used for further analysis.

Virus isolation and identification

Viruses were isolated in specific pathogen-free chicken embryonated eggs at 35℃ for 72 h. Allantoic fluid was harvested and an hemagglutination (HA) assay with 1% turkey red blood cells (TRBCs) was used to confirm the presence of influenza viruses. The HA positive samples were further subtyped by reverse transcription-polymerase chain reaction (RT-PCR) using primers published by Hoffmann[7].

Viral RNA extraction and RT-PCR

Viral RNA was extracted from allantoic fluid using an RNeasy Mini kit (Qiagen, Hilden, Germany) and transcribed into complimentary DNA (cDNA). The PCR reaction contained 3.0 μL cDNA, 1 μL forward primer and reverse primer, 12.5 μL RT-PCR Buffer, 1.25 μL Enzyme Mix, and 6.25 μL RNase-free water with a final volume of 25 μL. A single PCR program was used at 94℃ for 2 min, 35 cycles of denaturation at 94℃ for 30 s, annealing at 57℃ for 30 s, and extension at 72℃ for 3 min and 5 min.

Genome sequencing and phylogenetic analysis

The PCR products were visualized by agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced and the full genome sequences of the viruses were deposited in the Global Initiative on Sharing Avian Influenza Data (GISAID) database. Phylogenetic trees were generated using the neighbor-joining method with MEGA software (version 5.01, Molecular Evolutionary Genetics Analysis (MEGA; http://megasoftware.net/), and the bootstrap value was tested with 1000 replications for each gene.

Intravenous pathogenicity index (IVPI)

Stocks of the isolated virus were produced by passaging in 10-day-old SPF embryonated chicken eggs following a 10-fold serial dilution. To investigate its pathogenicity in chickens, 6-week-old SPF chickens in groups of 10 each were intravenously inoculated with 0.1 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid and housed in two isolator cages. Deaths were observed over a 10-day period.

Animal experiments

To investigate the pathogenicity in mammals, the virus was 10-fold serially diluted and each dilution was inoculated into six-week-old BALB/c mice (Beijing Vital River Laboratory Animal Technology Co.,Ltd.) at a dose of 50 μL per animal. The mice were monitored and weighed daily at two-week intervals. The mice to be euthanized were placed in a sealed box, and carbon dioxide was released to suffocate the mice. The lungs, nasal turbinates, spleens, and brains of the mice were pooled and the viral titers were determined.

Results

Real-time RT-PCR

Real-time RT-PCR showed that influenza A virus was positive (Figure 2A), and the H5, H7, and H9 subtypes were negative (Figure 1B–D).

Virus Isolation And Genome Sequencing

All samples were inoculated in 10-day-old SPF embryonated eggs, and the allantoic fluid was harvested after 72 h. The HI was approximately $2^8 - 2^9$. The isolated virus was named A/chicken/Taiwan/6634/2017 (i.e., TW6634). After basting with HA and NA sequences, TW6634 was found to be closely related to the H6N1 subtype.

Sequence Analysis

Table 1. The amino acid identities in A/chicken/Taiwan/6634/2017 (H6N1) and influenza viruses from the GenBank (National Center for Biotechnology Information

Gene	Closest strain	identity□%□
PB2	A/chicken/Taiwan/2267/2012(H6N1)	97.37
	A/chicken/PH/A2821/2013(H5N2)	97.72
PB1	A/chicken/Taiwan/2267/2012(H6N1)	96.83
	A/chicken/Taiwan/2593/2012(H5N2)	97.80
PA	A/chicken/Taiwan/2084/2012(H6N1)	97.72
	A/chicken/PH/A2821/2013(H5N2)	97.81
HA	A/chicken/Taiwan/2437/2012(H6N1)	96.71
NP	A/chicken/Taiwan/2759/2012(H6N1)	98.46
	A/chicken/Taiwan/8988/2013(H5N2)	98.60
NA	A/chicken/Taiwan/2084/2012(H6N1)	97.88
M	A/chicken/Taiwan/2267/2012(H6N1)	98.57
	A/chicken/Taiwan/2593/2012(H5N2)	98.27
NS	A/chicken/Taiwan/2084/2012(H6N1)	96.54
	A/chicken/Taiwan/A703-1/2008(H5N2)	96.54

The sequence analysis revealed that the TW6634 genes shared the highest nucleotide identity with the AIV subtypes prevalent in Taiwan province (Table 1) and indicated that the H6N1 virus from Taiwan province may have recombined from H5N2 and H6N1. The HA and NA genes of TW6634 shared a 96.71% and 97.88% identity with A/chicken/Taiwan/2437/2012 (H6N1) and A/chicken/Taiwan/2084/2012 (H6N1), respectively.

A bioinformatics analysis of the TW6634 viral genome revealed that the receptor binding sites of the HA geneof the TW6634 virus were P186, V190, Q226, and S228. This indicates that TW6634 can bind to the human α 2,6-sialic acid receptor. The NS1 gene had an Alanine mutation at locus 149, which suggested that it might improve the virulence of the virus in chickens [10]. The M2 gene had an Asparagine mutation at locus 31, suggesting that the virus might be resistant to amantadine [11].

Animal Experiments

After inoculating the virus into SPF chickens, all 10 chickens survived for 10 days, did not display any clinical signs, and the IVPI was 0.0, and then humanely euthanized with cervical dislocation. Thus, TW6634 is a LPAIV strain. After inoculating the TW6634 virus into BALB/c mice, none of the mice displayed any clinical signs and survived the entire observation period. Weight monitoring revealed that

only moderate transient weight loss occurred in the mice infected with a high dose of the virus (10^{-1} and 10^{-2} virus dilution groups) (Fig. 3). Three days after infection, four of the infected mice were sacrificed, and the lungs, sinuses, spleen, and brain tissues were collected for viral isolation and titration. The virus was isolated only in the lungs of infected mice with a titer of $10^{3.7}$ EID_{50}/g (Table 2).

Table 2 Survival and viral titer in animals infected with TW6634

virus	chicken		mouse	
	surval/tested	IVPI	surval/tested	Virus titer（log_{10} EID50/g）
TW6634	0/10	3.0	8/8	nasal turbinates（−）lung（3.70±0.50） brain（−）spleen（−）liver（−）

Discussion

In the present study, we isolated a LPAIV H6N1 strain from illegal frozen poultry seized from an international airplane. To further characterize the isolated virus, we conducted genome sequencing, phylogenetic analysis, and a pathogenicity evaluation. The IVPI of TW6634 was found to be 0.0, which is consistent with the amino acid sequence (PQIATR/G) at its HA cleavage site. The BLAST sequence analysis revealed that TW6634 displayed the highest sequence homology with the endemic H6N1 subtype of AIVs in Taiwan, with which it had the closest genetic relationship. The analysis of the HA receptor binding site showed that TW6634 could bind to the human α 2, 6-sialic acid receptor. In TW6634-infected mice, the virus was found to replicate in the lungs with weak pathogenicity and the mice that received a high dose of infection only exhibited moderate temporary weight loss. Influenza infections in poultry are of great concern due to the impact on both bird and public health, agricultural trade, and the high cost of associated control [12]. In June 2013, the first case of human avian H6N1 infection was reported in a Taiwanese woman[13].

Every year, a large number of illegally imported poultry products were confiscated in Shanghai. The illegally products include cooked eggs, chicken thigh meats, cooked chicken, cooked ducks and so on. This is the first time a complete frozen chicken include visceral was illegally imported by international passenger. The LPAI virus caused localized virus infections in respiratory and gastrointestinal tracts[14]. In this experiment, we collected samples from lung tissues may also increases the chances of detecting the virus.

International trade and movement, especially the illegal movement and smuggling of foods, are risk factors for the spread of infectious disease. The results of the present study indicate that it is necessary to further monitor international imports and exports. Moreover, H6N1 is a LPAIV commonly found in poultry and wild birds. The viral sequences isolated from poultry product shows that it is the closest to the Taiwan H6N1 virus strain native to poultry. Recently, the isolation rate of the AIV H6 subtype in China

has been increasing [15–17], and the infection rate of waterfowl is relatively high. Therefore, such rates may be related to the gradual enhancement of the adaptive ability of the waterfowl AIV H6 subtype to infect poultry [15]. Receptor binding tests show that more than one-third of the AIV H6 subtypes can recognize human receptors [17]. In addition, animal experiments have shown that some AIV H6 subtypes can effectively replicate in mice and be transmitted by contact [17]. Moreover, some studies have shown that the H6 virus can mutate into a HPAIV via mutations the sequence of the HA cleavage site, which results in sequences that are characteristic of HPAIVs. Therefore, the AIV H6 subtype is a potential threat to both humans and poultry.

Conclusion

In this study, we could not confirm the origin of poultry products and only able to trace the flight routine. However, the products illegally imported could be the origin of spreading the viruses and it is essential to increase publicity and awareness and strengthen inspection.

Declarations

Ethics Approval and Consent to Participate

In this research, all the studies using animals were submitted as protocols approved by the Animal Care and Ethics Committee of China Animal Health and Epidemiology Center (No.2017-25).All experiments were performed according to the guidelines of the committee. According to the Customs of PRC, the animal products were prohibited and we have the right to confiscate the product and detect them.

Consent to publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing Interest

The author declared that they have no conflict of interest.

Fuding

Not applicable.

Author’s contributions

WY designed the study and perfomed the experiments with JW and LJ, and analyzed the data together with ZL, ZQ and LC. WY wrote the initial draft of the manuscript and JW revised the manuscript. All

authors have read and approved the manuscript.

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Figures

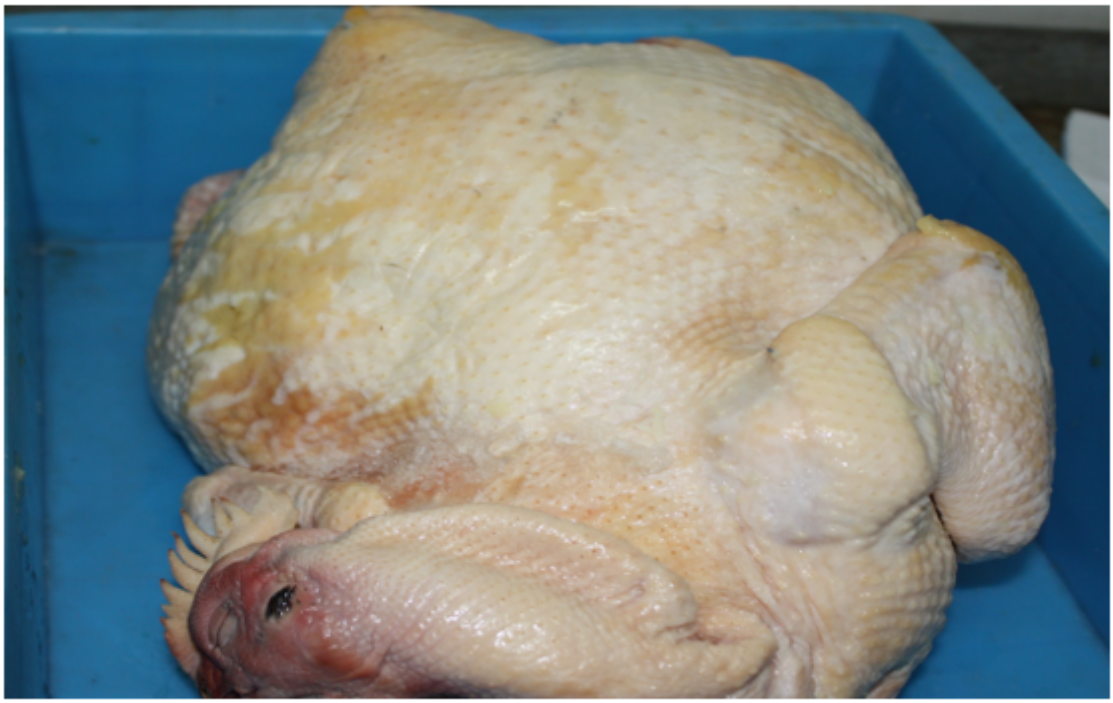

Figure 1

A frozen uncooked chicken.

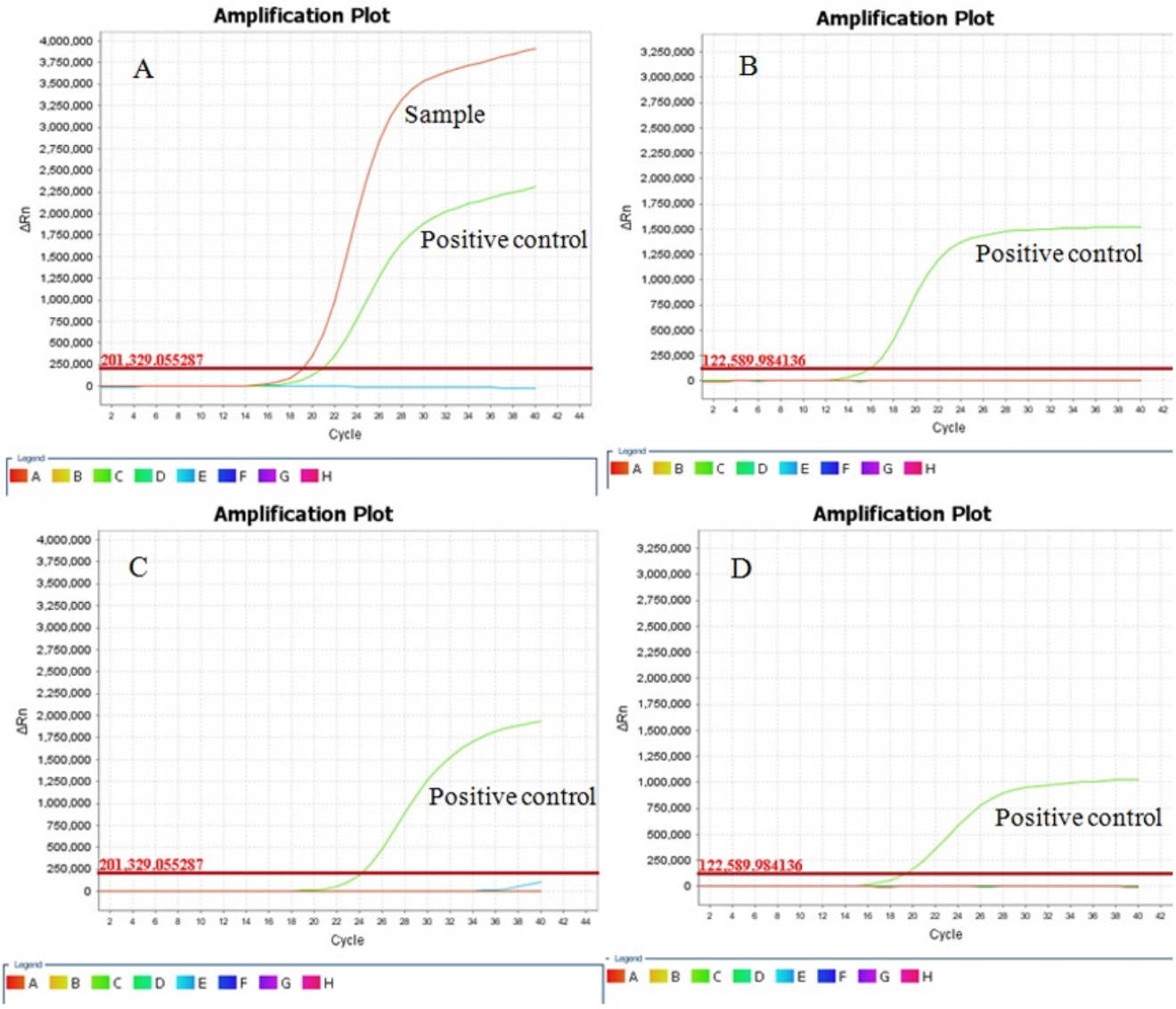

Figure 2

Real-time RT-PCR results. A. Influenza A assay; B. H5 subtype assay; C. H7 subtype assay; D. H9 subtype assay.

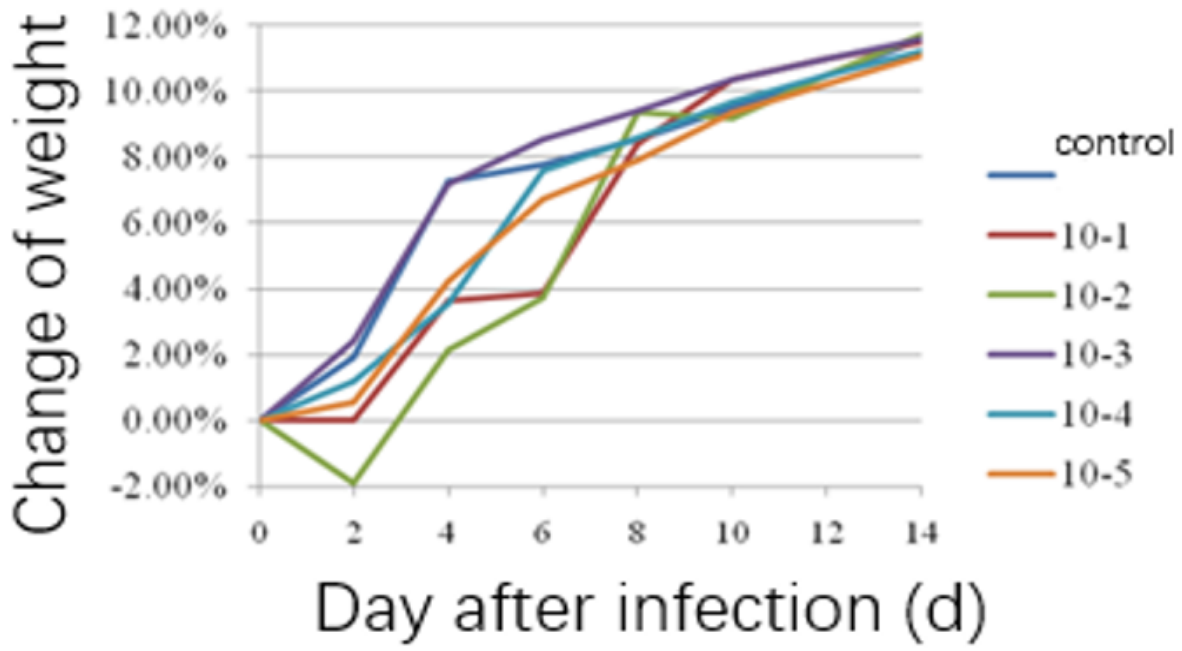

Figure 3

Weight change of mice infected with TW6634 virus.

Supplementary Files

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