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RESEARCH ARTICLE

Molecular Characterization and Pathogenicity Evaluation of a H1N1 Subtype Swine Influenza Virus

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A B S T R A C T

In order to investigate the prevalence of swine influenza virus (SIV) in Shandong, China, a pig farm in Binzhou, Shandong, was investigated. Nasal swab samples suspected of containing SIV were collected and inoculated into Specific Pathogen Free (SPF) chicken embryos for virus isolation. Subsequently, the isolated virus underwent whole-genome sequencing, genetic evolution analysis, protein key amino acid mutations analysis, and evaluation of pathogenicity to piglets. The results showed that a strain of SIV H1N1 subtype was successfully isolated and named as A/swine/Shandong/BZ03/2022 (H1N1), known as SIV H1N1 SDBZ strain. Sequence alignment analysis indicated that the HA and NA gene of the isolate exhibited the highest homology with A/swine/Liaoning/DL1007/2020 (H1N1) in GenBank, with similarities of 99.41 and 99.57%, respectively. The amino acid sequence of the HA protein cleavage site was identified as PSIQSR↓GLF, displaying low pathogenic molecular characteristics. The pathogenicity experiment demonstrated that infected piglets exhibited influenza symptoms such as fever, runny nose, and sneezing. SIV was consistently detected in nasal swabs of piglets, and led to meat-like consolidation in the lungs. Pathological examinations revealed parenchymal lungs with disappeared alveoli. Therefore, this study successfully isolated a H1N1 subtype SIV strain in Shandong Province, elucidating its genetic evolution, molecular characteristics, and pathogenicity. This work provides some important references for the monitoring and prevention and surveillance of SIV in China.

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INTRODUCTION

Swine influenza (SI) is an acute respiratory contagious viral disease caused by infection with swine influenza virus (SIV). It is characterized by respiratory symptoms, such as high fever, cough, sneezing, and increased nasal secretions. Pigs of all ages, particularly piglets, are susceptible to the virus. Some infected individuals may exhibit insignificant clinical signs, potentially being overlooked as source of infection, which can lead to persistent infection and outbreaks among pigs. While the mortality rate is typically low with a simple SIV infection, the presence of additional risk factors can increase mortality rates and result in significant economic losses for the pig breeding industry (Zhu *et al.,* 2011; Li *et al.,* 2022). SIV is classified under the genus Influenza A virus within the Orthomyxoviridae family and comprises eight single-stranded, negative-sense RNAs that encode both structural proteins (PB2, PB1, PA, HA, NP, NA, M) and nonstructural proteins (NS1) (Medina, 2011). SIV was first isolated from pigs in the United States in 1930. Subsequently, have been able to infect pigs across species and recombine with human-like H3N2 subtype influenza viruses, giving rise to various new viral strains. At present, the predominant global epidemic subtypes of SIV include H1N1, H1N2, and H3N2. Among these, the H1N1 subtype SIV includes classical H1N1 subtype (CS H1N1), Eurasia avian-like H1N1 (EA H1N1), human-like H1N1 (human H1N1) and 2009 pandemic H1N1 (pdm/09 H1N1) (Ito *et al.,* 1998; Zhao *et al.,* 2016; Russell *et al.,* 2018).

Porcine respiratory epithelial cells contain both a-2,3 galactose (SAa-2,3 Gal) and a-2,6 galactose (SAa-2,6Gal) receptors, facilitating their susceptibility to avian

influenza viruses and human influenza viruses. These cells serve as a natural "mixer" for genetic recombination, mutation and cross-species transmission of avian, human and porcine influenza viruses (Karasin *et al.,* 2000; Tilak *et al.,* 2021; Ozbek *et al.,* 2024). The recombination and mutation of influenza viruses from different species within pigs lead to significant economic losses in the swine industry and pose a public health threat to humans. Therefore, continuous surveillance of epidemic and mutant strains of SIV is crucial (Tilak *et al.,* 2021; Li *et al.,* 2022). In this study, a H1N1 subtype SIV strain was successfully isolated from a pig farm in Shandong Province, China. Through whole genome characteristics analysis and pathogenicity evaluation, the prevalence of SIV in the pig farms in Shandong was elucidated, providing a reference for the subsequent prevention and control of SIV.

MATERIALS AND METHODS

Animal ethics statement: The pathogenicity experimental was approved by the Animal Ethical and Welfare Committee of Sinovet (Jiangsu) Biopharmaceuticals Co., Ltd (No. HWT2023003) with the aim of minimizing the number of animals used.

Samples, chicken embryos: Nasal swab samples of sick pigs suspected of swine influenza at a large-scale pig farm in Binzhou, Shandong Province in November 2022. The samples subsequently preserved in DMEM (5ml) supplemented with penicillin G sodium (1000 IU/ml) and streptomycin sulfate (1000 μg/ml), and then transported to the laboratory under refrigerated conditions for storage at temperatures below -70°C. SPF chicken embryos (Jinan Saisi Poultry Technology Co., Ltd.) were utilized after reaching 9-11 days of incubation.

Experimental animals: Ten to twelve -week-old healthy and susceptible piglets, which tested negative for the antibody of Swine Influenza Virus (SIV) as well as the nucleic acid of SIV, African Swine Fever Virus (ASFV), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), and Pseudorabies Virus (PRV) were purchased from Jiangsu Langyue Biotechnology Co., Ltd.

Primer: Primers (Table 1) used in this study were newly synthesized by Nanjing Jinsirui Biotechnology Co., Ltd. according to (GB/T27521-2011 Swine Influenza Virus Nucleic Acid RT-PCR Detection method).

Sample Handling and Detection: The cryopreserved nasal swab samples were removed out from the ultra-low temperature and thawed at room temperature. Following thorough mixing by agitation, the liquid was transferred to another EP tube. Subsequently, 200μL of the solution was extracted for nucleic acid extraction as per the protocol of the nucleic acid extraction kit, and the swine influenza virus H1 subtype fluorescent Polymerase Chain Reaction (PCR) kit was employed for identification. In cases where positive results were obtained, the remaining liquid was subjected to centrifugation at 4 000 r/min for 10 minutes at 4℃. The resulting supernatant was filtered and sterilized using a disposable 0.45μm filter for further applications.

Table 1: Primer information

Primer pairs	Sequence	Fragment Length
H1-668U	5'-AGCAAAAGCAGGGGAAAATAA-3'	668 bp
H1-668L	5'-TGCATTCTGGTAGAGACTTTG-3'	
N1-615U	5'-TTGCTTGGTCRGCAAGTGC-3'	615 bp
N1-615L	5'-YCWGTCCAYCCATTWGGATCC-3'	

Virus isolation: The filtered liquid was inoculated into the allantoic cavity of 9 to 11-day-old SPF chicken embryos at a dosage of 0.2mL per embryo. Subsequently, the embryos were placed in an incubator at 37.5°C and 50% humidity for further cultivation. The chicken embryos that died after 24h of culture and those that had been cultured for 72h were placed at 4°C overnight. The allantoic fluid from the chicken embryo was collected under aseptic conditions. The hemagglutination titer (HA) of chicken red blood cells in the allantoic fluid was determined using micro-hemagglutination assay (Lu, 2019; Yang *et al.,* 2023). If the HA titer was equal to or greater than 1:8, the culture was expanded and characterized; In cases where the HA titer was less than <1:8, the culture was blindly propagated for two generations; If the HA titer remained below <1:8 after the second passage, the experiment was concluded.

Virus identification: The genotyping of SIV in the allantoic fluid was conducted using RT-PCR following the protocol outlined in the national standard (Lu, 2019). The virus liquid after expanded culture was sent to the third-party experimental service unit for electron microscope observation and shooting after ultrafiltration and chromatography purification. The identified SIV strain was named as A/swine/Shandong/BZ03/2022 (H1N1), referred to as SIV H1N1 SDBZ strain.

Determination of EID_{50}: The harvested virus solution underwent a 10-fold dilution with sterilized PBS (0.01 mol/L, pH 7.0~7.2), and five dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were taken. Each dilution was inoculated with four 9 to 11-day-old SPF chicken embryos, with each 0.1 ml of the diluted solution. The embryos were incubated at 37.0-38.0°C and 50-60% humidity, observations were made every 24 hours. The chicken embryos that died within 24h were abandoned, and the dead chicken embryos were removed after 24h to collect allantoic fluid, the remaining chicken embryos were taken out after 72h to collect allantoic fluid, and the hemagglutination (HA) titer of allantoic fluid was determined (Sun *et al.,* 2019; Yang *et al.,* 2023). If the HA titer was not less than 1:4, the infection was determined, and the EID_{50} titer of virus solution was calculated using Reed-Muench method (Reed *et al.,* 1938).

whole genome sequencing: The RNA of virus samples was extracted according to the kit's instructions. After the nucleic acid concentration is qualified, Shanghai Tanpu Biotechnology Co., Ltd. was entrusted to conduct whole genome sequencing utilizing the second-generation sequencing technology.

Genetic evolution and molecular characterization analysis: The submission of sequencing results of eight segments (PB2/PB1/PA/HA/NP/NA/M/NS) of SIV H1N1

SDBZ strain to NCBI for online BLAST alignment to identify the virus subtype and analyze nucleotide homology. Phylogenetic analysis of the gene sequences of the eight segments of the virus was performed using the adjacency method in MEGA7.0 (bootstrap 1000), with reference strain gene sequences obtained from the GenBank database. Additionally, potential glycosylation sites of HA and NA proteins were predicted using online software (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/), and the variation of key functional amino acid sites of the SIV H1N1 SDBZ strain was analyzed by MegAlign.

Pathogenicity experiment on piglets: According to Lu Wei's (Lu, 2019) experimental method for assessing SIV pathogenicity, seven healthy susceptible piglets aged 10-12 weeks were selected. Five piglets were randomly assigned to receive a tracheal injection of the SIV H1N1 SDBZ strain virus solution ($10^{5.5}EID_{50}$ / mL), with 4.0 mL administered to each piglet. The remaining two piglets were injected with sterile PBS as negative control. Temperature was monitored and clinical observations were conducted in the morning and afternoon daily during the 1-5 days post-challenge. Nasal swabs were collected every morning and afternoon during the 3-5 days post-challenge and inoculated into 9 to 11-day-old SPF chicken embryos, and if the HA titer was not less than 1:8, it was determined as SIV separation positive. On the 5th day post challenge, all piglets were euthanized and autopsied, and examined for lung lesions, and the lesion sites were collected for pathological analysis.

RESULTS

Virus isolation: The nasal swab samples collected were all found to be positive for H1 subtype SIV through RT-qPCR detection. The positive samples were inoculated into the allantoic cavity of chicken embryo for culturing purposes. The allantoic fluid from the chicken embryo was collected for erythrocyte agglutination test. The findings revealed hemagglutination in the allantoic fluid of three samples had, with sample No. 03 exhibiting the highest HA titer of 1:128. The virus liquid from sample No. 03 was inoculated into allantoic cavity of 9 to 11-day-old SPF chicken embryo for expanded culture. The HA titer of the resulting allantoic fluid was determined to be 1:256, and the infection titer was $10^{5.5}$ EID_{50}/mL.

Virus identification: The RT-PCR results showed that the isolated virus successfully amplifies specific bands corresponding to the H1 and N1 genes (Fig. 1). Analysis through transmission electron microscopy showed that the diameter of the virus particles was 100 ± 20 nm, spherical or oval, with a capsule, and radial protrusions were observed on the surface of the capsule (Fig. 2). According to the international naming conventions, the isolated virus strain was named as A/swine/Shandong/BZ03/2022 (H1N1), referred to as SIV H1N1 SDBZ strain.

Whole-genome homology alignment: The sequencing results showed that the eight gene segments of SIV H1N1 SDBZ strain exhibited the highest homology with the corresponding gene segments of seven influenza virus strains from various years and regions. Among these, PB2, PB1, PA, HA, NP, NA and M genes displayed the highest homology with H1N1 subtype SIV, while the NS gene exhibited the highest homology with H3N2 subtype SIV (Table 2). The results showed that SIV H1N1 SDBZ strain may be consist of the gene fragments derived from different swine influenza virus strains, and each gene fragment displaying varying degrees of variation.

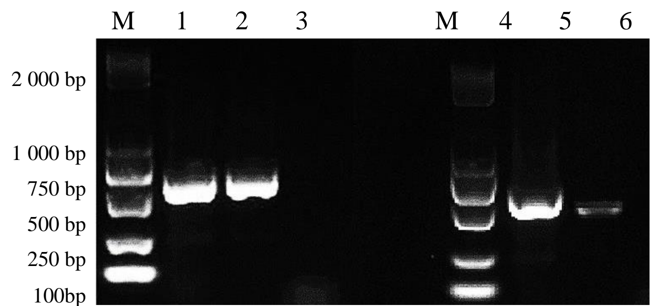


Fig. 1: RT-PCR identification results of H1 and N1 genes of isolates. M: DL2000Maker; 1: Isolate H1 gene; 2: SIV H1 gene positive control (668bp); 3: Negative control; 4: Isolate N1 gene; 5: SIV N1 gene positive control (615bp); 6: Negative control.

Table 2: Homology of SIV H1N1 SDBZ gene to other isolates

Gene	Isolate strain ID	Homology	Accession
PB2	A/swine/Liaoning/PJ89/2014(H1N1)	97.11%	MN393805.1
PB1	A/swine/Liaoning/CY102/2014(H1N1)	97.27%	MN393726.1
PA	A/swine/Liaoning/AS1732/2020(H1N1)	98.69%	OL311409.1
HA	A/swine/Liaoning/DL1007/2020(H1N1)	99.41%	OL310829.1
NP	A/swine/Tianjin/42/2011(H1N1)	96.93%	KP404405.1
NA	A/swine/Liaoning/DL1007/2020(H1N1)	95.60%	OL310931.1
M	A/swine/Tianjin/184/2011(H1N1)	97.97%	KP336281.1
NS	A/swine/Guangxi/NS1402/2012(H3N2)	97.37%	KM028483.1

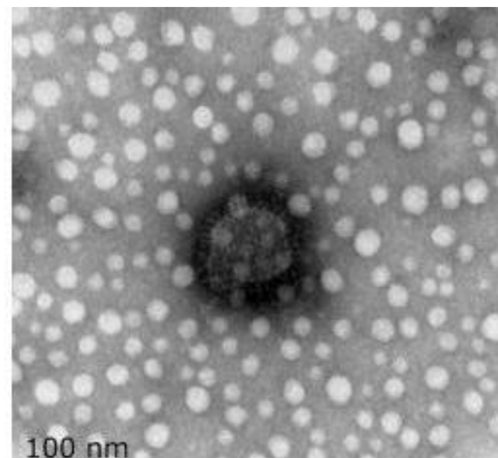
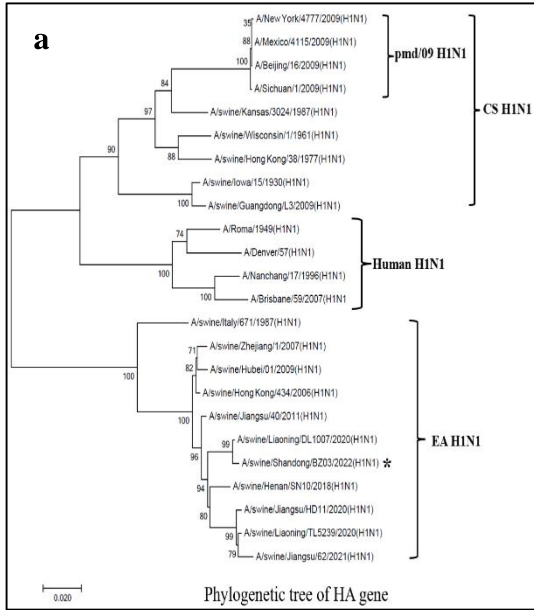
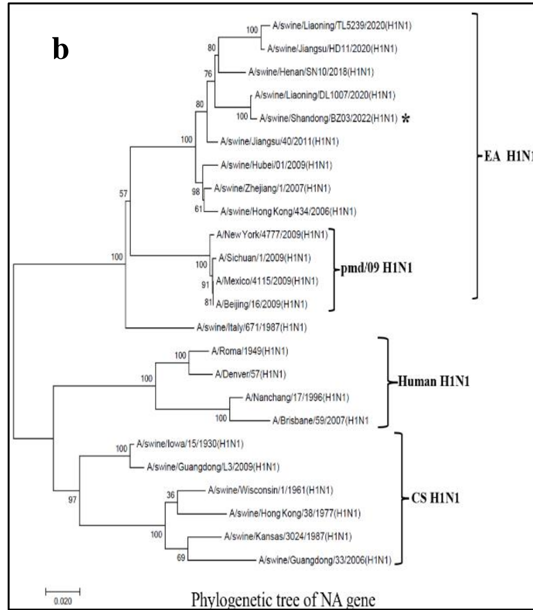


Fig. 2: Electron microscope picture of isolated virus. The virus particles were spherical or oval, with a capsule, and radial protrusions were observed on the surface of the capsule.

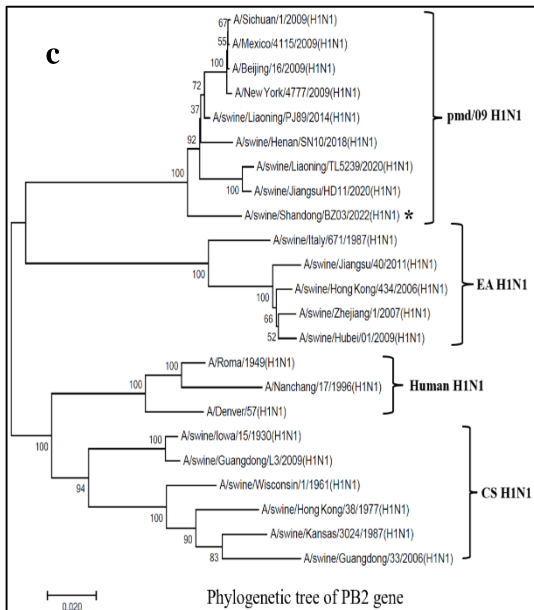
Genetic and evolutionary analysis of the whole gene: The genetic phylogenetic tree analysis showed that the HA and NA genes of SIV H1N1 SDBZ strain were derived from new sub-branch of EA H1N1 lineage (Fig. 3a, Fig. 3b), the internal genes PB2, PB1, PA, NP and M genes of SIV H1N1 SDBZ strain were derived from the 2009 pandemic H1N1 lineage (pdm/09 H1N1) (Fig. 3c-Fig. 3g), and NS gene was derived from the classical H1N1 lineage (CS H1N1) (Fig. 3h).



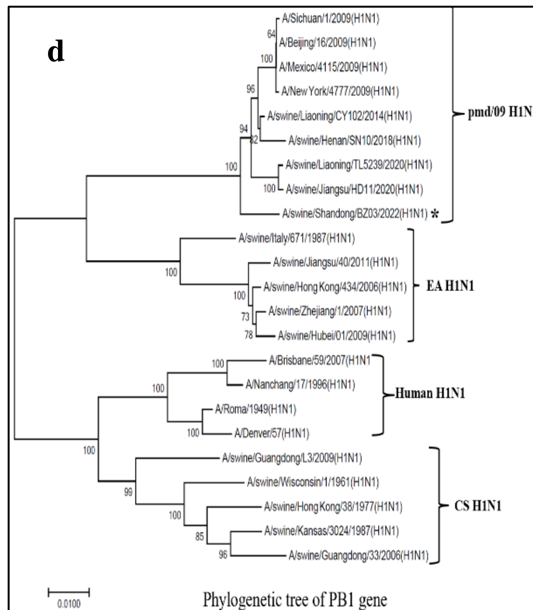
Phylogenetic tree of HA gene



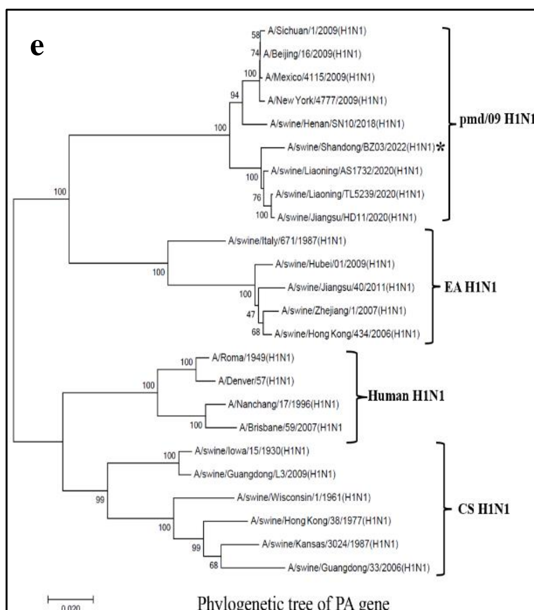
Phylogenetic tree of NA gene



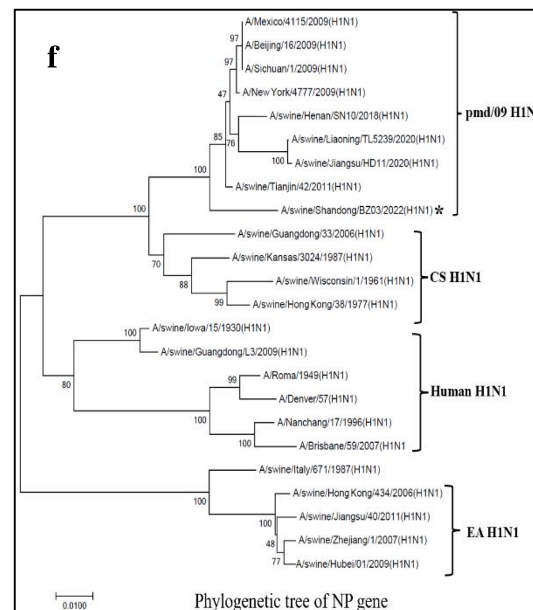
Phylogenetic tree of PB2 gene



Phylogenetic tree of PB1 gene



Phylogenetic tree of PA gene



Phylogenetic tree of NP gene

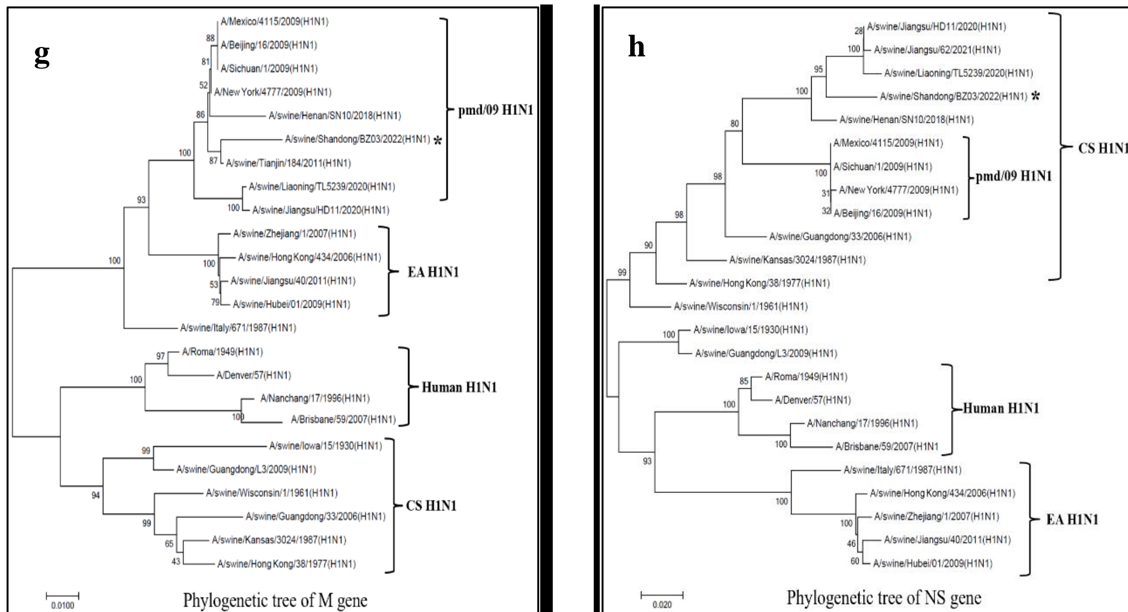


Fig. 3: Genetic Phylogenetic Tree of (a) HA Gene, (b) NA Gene, (c) PB2 Gene, (d) PB1 Gene, (e) PA Gene (f) NP Gene, (g) M Gene, and (h) NS Gene of SIV H1N1 SDBZ Strain (*). HA gene and NA gene belong to EA H1N1 lineage, NS gene belongs to CS H1N1 lineage, and the other five genes belong to the pdm/09 H1N1 lineage.

Analysis of viral molecular characteristics: Based on the nucleotide sequences of the HA and NA genes, the amino acid sequences were deduced. SignalP 4.0 was utilized to predict that the signal peptide sequence of the HA protein was located at amino acid position 1 to 17. Through Lasergene analysis, it was determined that the amino acid sequence of the HA protein of SIV H1N1 SDBZ strain at the cleavage site was PSIQSR↓GLF, aligning with the molecular characteristics of low pathogenicity influenza virus (Zhao *et al.,* 2016). The online predictions from NetNGlyc-1.0 showed that the HA protein had five potential glycosylation sites, while the NA protein had seven potential glycosylation sites (Table 3). Mutations E190D and G225E was identified in the receptor binding sites (RBS) of the HA protein, indicating their potential to bind to human sialic acid receptor (Wang *et al.,* 2017). No mutations were found in the amino acids 119E, 199D, 223I, 275H, 293R, and 295N of the NA protein, indicating that SIV H1N1 SDBZ strain maintained a high sensitivity to neuraminidase inhibitors (Huang *et al.,* 2020). Analysis of key amino acid sites in internal proteins showed that SIV H1N1 SDBZ had amino acid sites mutations related to SIV pathogenicity, virulence, and enhanced replication ability (Zhu *et al.,* 2015; Zhang *et al.,* 2023), such as V171M, R198K, N375S, and L473V mutations in the PB1 protein, S31N mutation in the M2 protein, and G149A mutation in the NS protein (Table 4).

Pathogenicity experiment on piglets: Between 2 to 5 days post-challenge (dpc), all piglets in the infected group presented one or more of the respiratory symptoms, such as fever (>40.2 °C), sneezing, and coughing, whereas no abnormalities were observed in the control group. Between 3 to 5 days post-challenge, the HA titers of the chicken embryo cultures derived from the nasal swabs of the infected group were consistently at or above 1:32, indicating that the infected piglets could excrete toxins to the external environment through the nasal cavity. On the 5th day post-challenge post-mortem examinations of all animals showed that the lungs of the infected piglets displayed plaque-like fleshy consolidation (Fig. 4a), while those of the control group appeared normal (Fig. 4b). Histopathological analysis demonstrated extensive consolidation in the lung tissues of the infected group, with a blurred, atrophied, or even absent alveolar structure (Fig. 5a), in contrast to the control group where no consolidation was observed, and the alveolar structure remained intact (Fig. 5b). In conclusion, the SIV H1N1 SDBZ strain has strong pathogenicity to piglets.

Table 3: Analysis of HA and NA glycosylation sites

Gene	Position	Potential	Jury agreement	N-Glyc result
	28	NSTD	0.7960	+++
	40	NVTV	0.7512	+++
HA	291	NCTT	0.5112	+
	498	NGTY	0.5152	+
	557	NGSL	0.6776	++
	44	NQSE	0.6676	+
	58	NNTW	0.5281	+
	63	NQTY	0.6824	++
NA	68	NVSN	0.7658	+++
	88	NSSL	0.7369	++
	146	NGTV	0.6612	++
	235	NGSC	0.7320	++

DISCUSSION

At present, swine influenza has emerged as a global epidemic, presenting a significant challenge to the prevention, control and public health safety in pig farming. Ten different subtypes of influenza viruses have been identified globally, including H1N1, H1N7, H2N3, H3N1, H3N2 and H3N3. In China's pig population, the Eurasian avian-like H1N1 subtype SIV is predominant (Xiao *et al.,* 2019). In recent years, the lasting impact of African Swine Fever (ASFV) has led to a shift China's pig farming model practices towards intensive and large-scale development, which makes it easier to implement biosecurity measures. Stringent biosecurity measures can

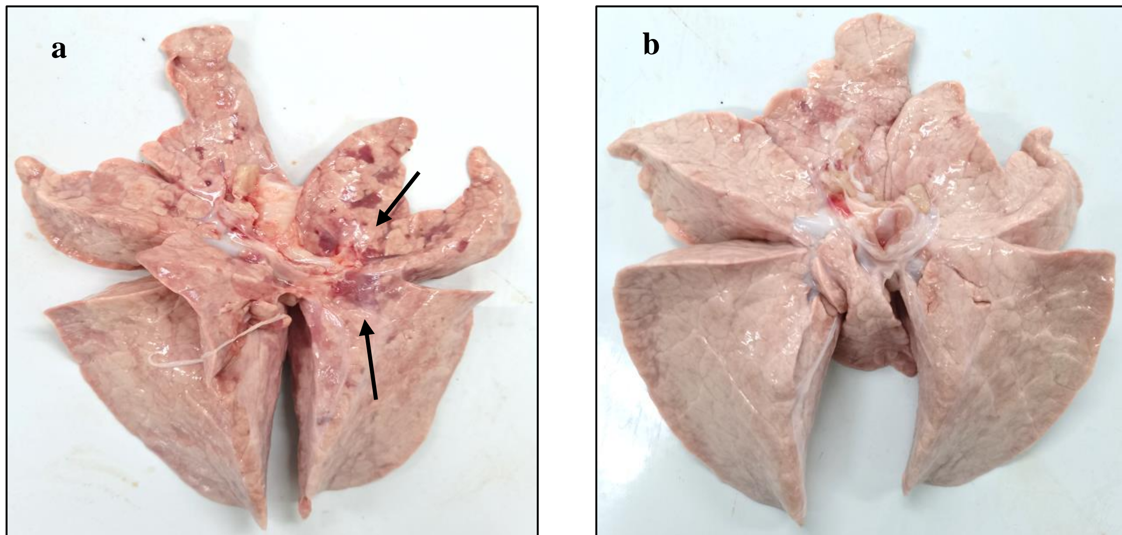


Fig. 4: General pathological changes of the lung. (a) The lungs of infected piglets showed plaque-like fleshy consolidation, were indicated by arrows. (b) The lungs of control piglets were normal.

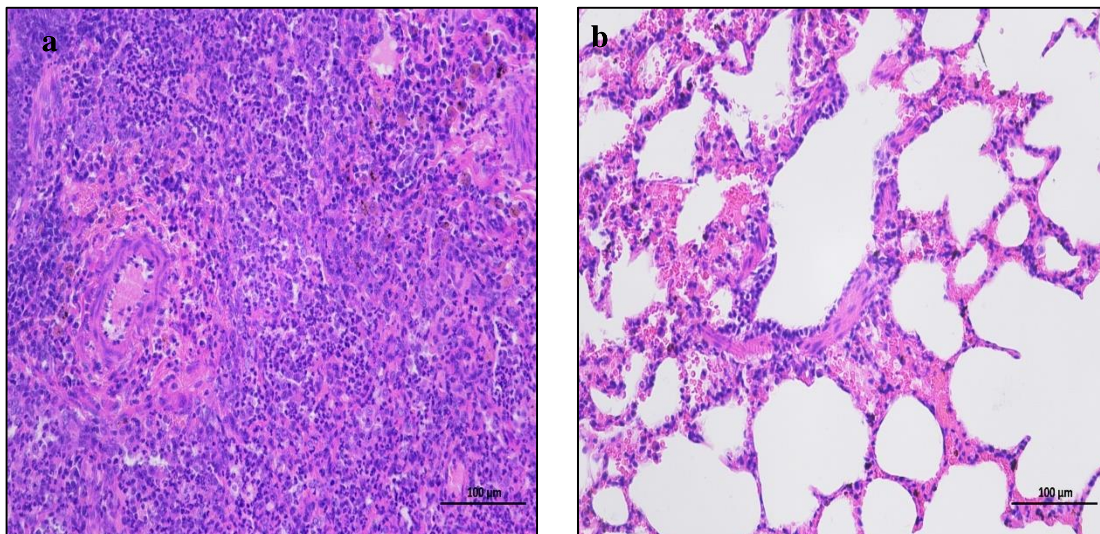


Fig. 5: Lungs of the infected piglets (a) and the control piglets (b) stained by hematoxylin and eosin (HE). (a) The lungs of infected piglets showed alveolar disappearance and pulmonary parenchyma. (b) The lungs of control piglets were normal. The structure of lung alveoli of piglets in the control group is clear.

Table 4: Mutation analysis of functional amino acid sites of SIV H1N1 SDBZ

Gene	Mutation	Function	SIV H1N1 SDBZ
PB2	E627K、D701N	Enhance the virulence of influenza virus.	-
PB1	H99Y, V171M, R198K, T296R M317I, N375S, L473V, L598P	Enhance the virulence of influenza virus.	171M, 198K, 375S, 473V
PA	P224S	Enhance the virulence of influenza virus.	224S
HA	E190D、G225E	Determine the host specificity of influenza virus	190D，225E
NP	K319N	Enhancing the pathogenicity of influenza virus in mice.	319N
NA	E119V, R152K, H275K, R293K, N295S	Mutations in resistance to neuraminidase inhibitors.	-
M2	L26F, V27A, A30T, S31N	Mutations in resistance to amantadine and amantadine.	S31N
NS	D92E, G149A	Enhance the virulence of influenza virus.	149A
	A42S	Promote the replication of influenza virus.	42S

Note: "-" No mutation.

mitigate the impact of certain diseases like African swine fever, but they are not effective in preventing and controlling swine influenza. This is due to the multiple modes of transmission of SIV, including through air droplets, direct contact and airborne aerosols, with the enclosed and centralized farming model providing conducive environments for its dissemination. SIV infection in pigs can result in damage to respiratory epithelial cells, potentially leading to secondary bacterial infections such as *Haemophilus parasuis*, *Streptococcus* and *Pasteurella*, as well as concurrent viral infections like PRRSV and PCV (Wang *et al.,* 2017; Xiao *et al.,* 2019). Coexistence of different SIV subtypes of in pigs may facilitate mutations or genetic recombination, altering their pathogenicity and genetic makeup, thereby increasing the risk of a swine influenza pandemic (Zhao *et*

al., 2021). Therefore, conducting epidemiological investigation and isolating the SIV virus to master the genetic variations, are crucial steps in developing effective prevention and control measures for swine influenza.

The diversity of influenza virus receptor hosts is intricately linked to the amino acids of receptor binding sites within the HA protein. The amino acids at position 190 and 225 of the HA protein play a key crucial role in the receptor binding to the host cell and are pivotal in the adaptation process of avian influenza virus to mammalian hosts, such as pigs and humans (Feng *et al.,* 2022). The key amino acid sites of the HA protein of SIV H1N1 SDBZ strain are 190D and 225E, indicating that the virus can bind to human sialic acid receptor (SA-a-2,6-Gal) in addition to avian sialic acid receptor (SA-a-2,3-Gal) (Tumpey *et al.,* 2007; Yang *et al.,* 2016). The cleave site PSIQSR↓GLF within the HA protein of SIV H1N1 SDBZ strain aligns with the molecular characteristics of low pathogenicity SIV (LPSIV). However, experimental evidence presented in this study demonstrates that SIV H1N1 SDBZ strain can induce typical influenza symptoms in healthy susceptible piglets post infection, with significant consolidation in the lungs, indicating that the isolate has high pathogenicity. This heightened pathogenicity may be attributed to additional mutations beyond those in the HA protein, including V171M, R198K, N375S and L473V mutations in the PB1 protein, P224S mutation in the PA protein, and G149A mutation in the NS protein, which could potentially enhance the virulence of SIV H1N1 SDBZ (Zhu *et al.,* 2015; Gong *et al.,* 2015; Prokopyeva *et al.,* 2016; Cheng *et al.,* 2020).

Conclusions: In summary, a SIV strain with H1N1 subtype was isolated from Shandong, China and named as A/swine/Shandong/SDBZ/2022(H1N1). The isolate virus was derived from the EA H1N1 lineage and exhibited high pathogenicity in piglets. The results of this study provide some important data for the epidemiological monitoring of SIV in pigs in China, and serve as a reference for the prevention and control of swine influenza.

Competing interests: The authors declare that they have no competing interests.

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Authors contributions: LT and CC conceived and designed the study. CC, CT, SX and YZ executed the experiment and analyzed the clinical samples. CT and LT analyzed the data. Each author interpreted the data, critically revised the manuscript and approved the final version.

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