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Electronic Supplementary Material

Human H9N2 Avian Influenza Infection: Epidemiological and Clinical Characterization of 16 Cases in China

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Supplementary materials

METHODS

Patients and data collection

Inpatients that presented with influenza-like illness at Wuhan JinYinTan Hospital in Wuhan (China) between January and February 2019 were included in this study. The history of hospitalization and physical examination, hematological, biochemical, radiological, and microbiological test results were collected.

Samples and data collection

Oropharyngeal swabs from patients with influenza-like illness were detected by rRT-PCR and colloidal gold kits, and fifty-four sawbs with influenza A-positive were subjected to next-generation sequencing (NGS) at the end of April 2019. In total, 16 H9N2 complete genomes were obtained. After laboratory confirmation, we retrospectively studied epidemiological, clinical, and virological characteristics of the patients. Seven patients were tracked and 13 serum samples were collected from these patients and their family members.

Laboratory tests

Oropharyngeal swabs collected on admission were analyzed for the presence of influenza A/B viruses and other respiratory viruses using real-time reverse-transcription (rRT)-PCR assay kits (Z-RR-0097-02; Shanghai Zhijiang Biotechnology) and colloidal gold kits (SD Bioline Influenza Ag; Standard Diagnostics). Complete genome sequences of influenza A virus in samples were obtained by NGS. AIV H9N2 human infections were confirmed by RT-PCR with H9-specific primers (Bi *et al*. 2016), virus isolation, and hemagglutination inhibition (HI) assays.

Virus isolation and HI assay

Orophyaryngeal swabs were inoculated into specific pathogen-free chicken embryos to isolate the virus. The H9N2 isolates were confirmed by hemagglutination assay and RT-PCR. HI assays were conducted using chicken erythrocytes according to WHO guidelines, with modifications reported by Gregory et al (Huang *et al*. 2013; Kayali *et al*. 2008; World Health Organization. 2011).

Statistical analysis

The independence test between the clinical characteristics and severe/mild cases and death in severe cases were done by using Fisher's exact test. All analyses were performed using SPSS software for Windows (version 17.0).

Definitions of human H9N2 infection, sepsis, acute respiratory distress syndrome (ARDS), and severe influenza

Section 1. Human H9N2 case definitions

The case definitions of confirmed human infection with H9N2 AIVs were based on the H5N1 case definitions, according to World Health Organization (WHO) in 2006 (World Health Organization. 2006). In this study, the oropharyngeal swabs from influenza-like illness (ILI) patients which were influenza A nucleic acid or antigen positive were sequenced by next generation sequencing (NGS). The whole genome of Avian influenza H9N2 virus were found in the oropharyngeal swabs of patients, subsequently, human cases with H9N2 avian influenza infection were confirmed by RT-PCR with H9 specific primers, virus isolation, and convalescent patients' sera (tested by haemagglutination inhibition assays) (World Health Organization. 2011)

Section 2. Definitions of sepsis (Singer *et al*. 2016)

The sepsis was diagnosed based on definitions in Sepsis 3.0. The patients have at least 2 of the following clinical features of quickSOFA (qSOFA): respiratory rate of 22/min or greater, altered mentation, or systolic blood pressure of 100 mm Hg or less.

Section 3. Definitions of acute respiratory distress syndrome (ARDS) (Force *et al*. 2012)

ARDS was diagnosed according to ARDS Berlin definitions, namely:

(1). Timing: within 1 week of a known clinical insult or new or worsening respiratory symptoms

(2). Chest imaging: bilateral opacities—not fully explained by effusions, lobar/lung collapse, or nodules

(3). Origin of edema: respiratory failure not fully explained by cardiac failure or fluid overload need objective assessment (eg, echocardiography) to exclude hydrostatic edema if no risk factor present

(4). Oxygenation: Mild: 200 mm Hg < PaO2/FIO2 ≤ 300 mm Hg with PEEP or CPAP ≥ 5 cm H2O Moderate: 100 mm Hg < PaO2/FIO2 ≤ 200 mm Hg with PEEP ≥ 5 cm H2O Severe: PaO2/FIO2 ≤ 100 mm Hg with PEEP ≥ 5 cm H2O

Section 4. Definitions of severe influenza case

The severe influenza case was diagnosed according to Management For Diagnosis and Treatment of Influenza revised edition (2018) in China (National Health Commission of the People's Republic of China. 2018), one of following situations occurs:

(1). Persistent fever > three days, associated with intense and rapid coughs, purulent sputum, hemoptysis and chest pain

(2). Increasing respiratory rate, dyspnea, cyanosis

(3). Altered mental status: slow reaction, drowsiness, restlessness, convulsions

(4). Severe vomit, diarrhea, dehydration

(5). Complicated pneumonia
(6). Deterioration of comorbid disease

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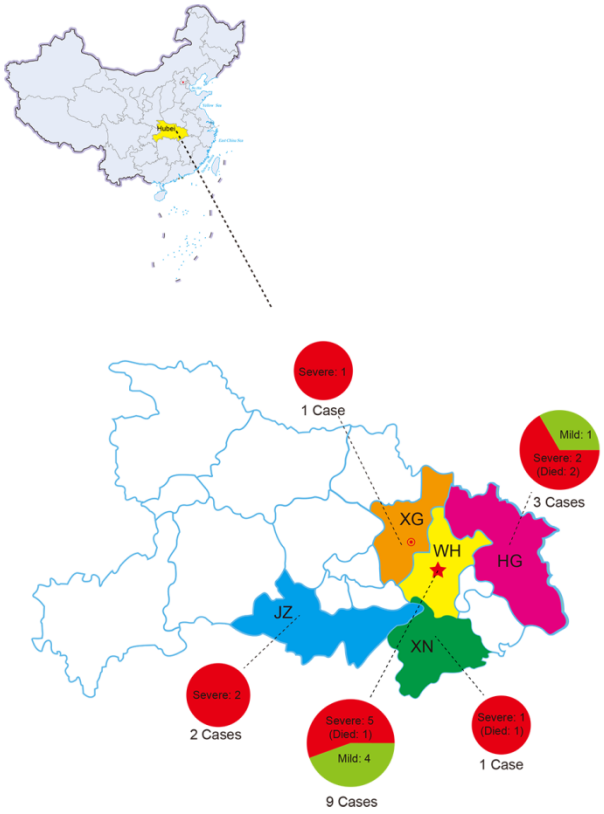

Fig. S1. Geographic distribution of 16 confirmed H9N2 cases in Hubei Province, China in 2019. Human cases of infection with H9N2 avian influenza virus distributed in five cities: Jingzhou (JZ, cyan); Xiaogan (XG, orange); Wuhan (WH, yellow); Huanggang (HG, magenta); Xianning (XN, green).

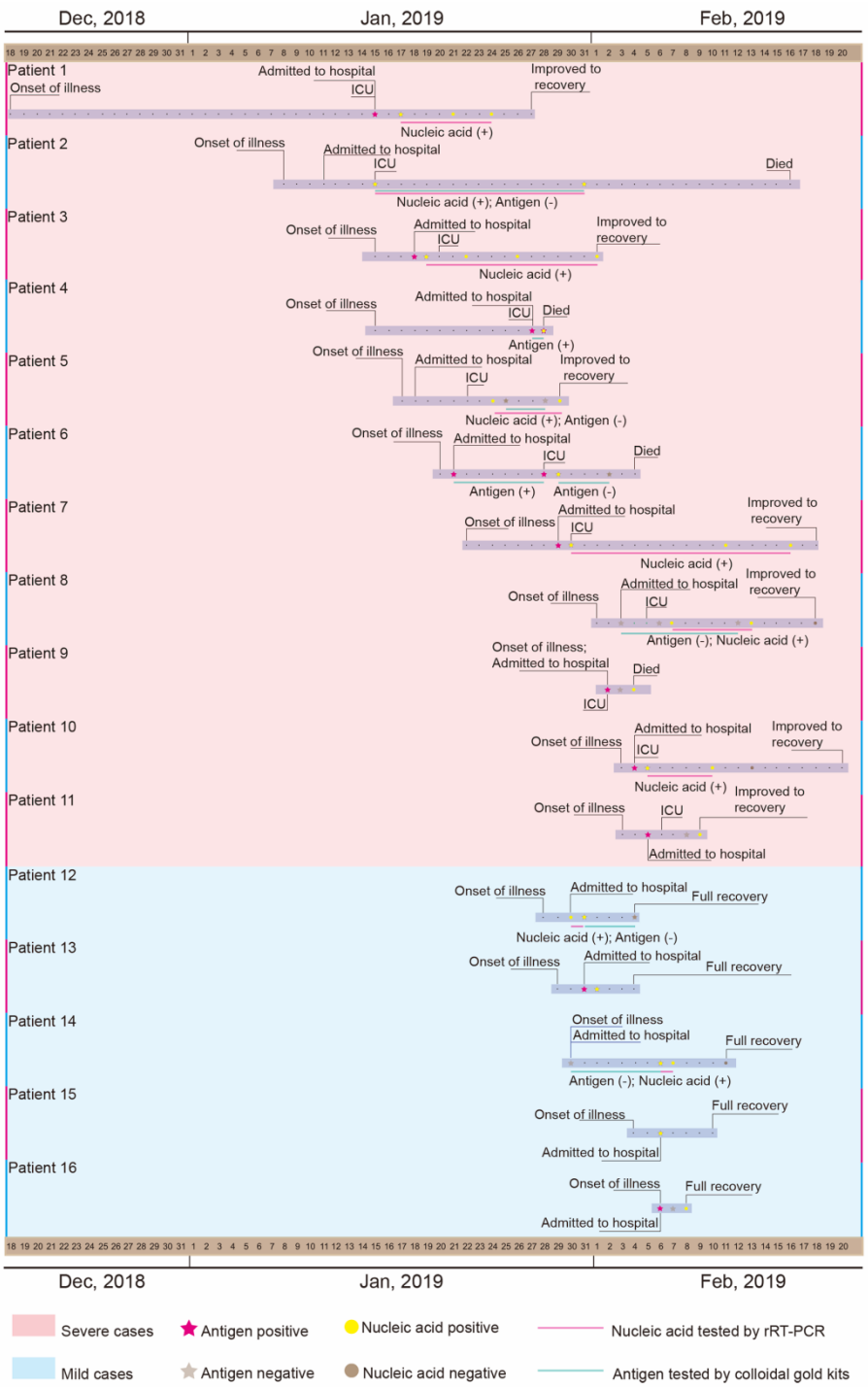

Fig. S2. Timeline of the disease course of the 16 patients studied. In total, 16 patients were included in this study, including 11 severe cases (patients 1 to 11, pink background, upper part) and 5 mild cases (patients 12 to 16, blue background, lower part). Abbreviation: ICU, intensive care unit.

Table S1. Patients, related to Figure S1.

Patient	Sex	Age (years)	Severity of illness	Location	Outcome
Patient 1	Male	61	Severe	Xiaogan	Improved to recovery
Patient 2	Male	46	Severe	Huanggang	Died
Patient 3	Female	62	Severe	Wuhan	Improved to recovery
Patient 4	Male	48	Severe	Huanggang	Died
Patient 5	Female	75	Severe	Wuhan	Improved to recovery
Patient 6	Male	88	Severe	Wuhan	Died
Patient 7	Male	62	Severe	Jingzhou	Improved to recovery
Patient 8	Male	62	Severe	Wuhan	Improved to recovery
Patient 9	Male	55	Severe	Xianning	Died
Patient 10	Female	60	Severe	Jingzhou	Improved to recovery
Patient 11	Male	82	Severe	Wuhan	Improved to recovery
Patient 12	Female	68	Mild	Wuhan	Full recovery
Patient 13	Male	68	Mild	Wuhan	Full recovery
Patient 14	Female	1.1	Mild	Wuhan	Full recovery
Patient 15	Male	2.1	Mild	Huanggang	Full recovery
Patient 16	Female	48	Mild	Wuhan	Full recovery

Note: The median age of these 16 patients was 61.5 years.

Table S2. Haemagglutination inhibition (HI) antibody titres against avian influenza virus H9N2*.

Sera[†]	HI titres (anti-H9N2 AIV)[§]
Patient 1	40
Patient 7	80
Patient 8	160
Patient 10	80
Patient 13	160
Patient 16	160

Patient 1-W	160
Patient 4-W	80
Patient 4-S	40
Patient 7-W	80
Patient 8-W	80
Patient 13-W	80
Patient 16-H	80

*A total of 13 sera were collected from six patients and seven family members, about five months after they discharged from hospital. Avian influenza virus H9N2: A/chicken/Hunan/06.22 YYGK2T3/2018(H9N2).

†W: patient's wife; S: patient's son; H: patient's husband.

§ Serum samples were considered positive if titers ⩾ 1:80, and all results were from at least 2 independent assays.

Table S3. Clinical features, laboratory results, and treatment of five mild cases.

Characteristic	Value
Age (years)	
Median (range)	48 (1.1–68)
Subgroup — no. (%)	
0–4	2 (40)
5–14	0
15–49	1 (20)
50–64	0
≥65	2 (40)
Male — no. (%)	2 (40)
Transferred to ICU* — no. (%)	0
Underlying health conditions — no. (%)	0
Onset symptom — no. (%)	
Fever	5 (100)
Cough, expectoration, chest distress	2 (40)

Diarrhea or vomit	1 (20)
Selected Laboratory abnormalities — no. (%)	
Increased WBC†	1 (20)
Neutrophilia	3 (60)
Lymphopenia	3 (60)
Chest radiologic findings — no. (%)	
Normal	1 (20)
Increased bronchovascular shadows	4 (80)
Clouding opacity	4 (80)
Treatment — no. (%)	
Antiviral treatment (oseltamivir)	5 (100)
Oxygen therapy (nasal catheter)	2 (40)
Antibiotic treatment	4 (80)
Clinical outcome — no. (%)	
Full recovery	5 (100)

*ICU: intensive care unit.

†WBC: white blood cell count.

Table S4. Clinical characteristics, infection indicators, treatment, and clinical outcomes of 5 mild cases.

Characteristic	Patient 12	Patient 13	Patient 14	Patient 15	Patient 16
Age (years)	68	68	1.1	2.1	48
Sex	Female	Male	Female	Male	Female
Occupation	Retired	Retired	Infant	Infant	Self-employed
Comorbid conditions	No	No	No	No	No
Onset of symptom	Fever, cough, expectoration, and chest distress	Fever	Fever	Fever and vomit	Fever, cough, expectoration, chest distress, and dyspnea
Date of illness onset	2019.01.28	2019.01.29	2019.01.30	2019.02.04	2019.02.06
Date of admission	2019.01.30	2019.01.31	2019.02.06	2019.02.05	2019.02.06
Transferred to ICU*	No	No	No	No	No
Date of specimen collected to be sequenced	2019.01.31	2019.02.01	2019.02.07	2019.02.06	2019.02.08
Spesis	No	No	No	No	No
ARDS†	No	No	No	No	No
WBC§ ($\times 10^9$/L)	5.8	6.18	13.79	8.78	5.74
L (%)§	9.4	7.5	52.2	15.9	7.3
N (%)§	81	85.8	31	70.1	90.3
PCT (ng/ml)§	0.1	<0.05	<0.05	NA	<0.05

Sputum cultureof G+/G-‡	(-)	(-)	(-)	(-)	NA
Oxygen therapy	Nasal catheter	No	No	No	Nasal catheter
Antiviral agent	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir
Interval of illness onset to antiviral therapy (days)	2	2	7	2	1
Duration of antiviral therapy (days)	5	4	5	4	2
Stay in hospital (days)	5	5	5	4	1
Outcome	Full recovery	Full recovery	Full recovery	Full recovery	Full recovery

Note: (-) indicates negative; NA: not applicable.

*ICU: intensive care unit.

†ARDS: acute respiratory distress syndrome.

§ WBC: white blood cells count; L (%): percent of lymphocyte; N (%): percent of neutrophile granulocyte; PCT (ng/ml): procalcitonin.

‡Sputum culture of G+/G: culture and identify Gram-positive/Gram-negative bacterica in sputum.

Table S5. Clinical features, laboratory results, and treatment of 11 severe cases.

Characteristic	Value
Age (years)	
Median (range)	62 (46–88)

Subgroup — no. (%)	
0–4	0
5–14	0
15–49	2 (18.2)
50–64	6 (54.5)
≥65	3 (27.3)
Male — no. (%)	8 (72.7)
Transferred to ICU* — no. (%)	11 (100)
Underlying health conditions — no. (%)	9 (81.8)
Cardiovascular diseases	5 (45.5)
Respiratory diseases	2 (18.2)
Endocrine system diseases	2 (18.2)
Central nervous system diseases	1 (9.1)
Digestive system diseases	2 (18.2)
Urinary system diseases	1 (9.1)
Onset symptom — no. (%)	
Fever	9 (81.8)
Cough, expectoration, chest distress, dyspnea	11 (100)
Bloody sputum	2 (18.2)
Muscle ache	1 (9.1)
Selected laboratory abnormalities — no. (%)	

Sepsis	10 (90.9)
ARDS[†]	7 (63.6)
Neutrophilia	11 (100)
Lymphopenia	11 (100)
Increased procalcitonin	9 (81.8)
Bacteria or fungus isolation from culture	3 (27.3)
Bacteria	2 (18.2)
Fungus	3 (27.3)
Chest radiologic findings — no. (%)	
Involved bilaterally	11 (100)
Increased bronchovascular shadows	11 (100)
Clouding opacity	11 (100)
Widely distributed flocculent, and ground-glass opacity	3 (27.3)
Treatment — no. (%)	
Antiviral treatment (oseltamivir)	11 (100)
Oxygen therapy	
Mechanical ventilation	3 (27.3)
Mask	2 (18.2)
Nasal catheter	3 (27.3)
High flow oxygen	3 (27.3)
Antibiotic treatment	

Combined use of multiple antibiotics	11 (100)
Antifungal therapy	3 (27.3)
Glucocorticoid therapy	2 (18.2)
Intravenous immune globulin therapy	3 (27.3)
Clinical outcome — no. (%)	
Deceased	4 (36.4)
Improved to recovery	7 (63.6)

*ICU: intensive care unit

†ARDS: acute respiratory distress syndrome

Table S6. Clinical characteristics, infection indicators, treatment, and clinical outcomes of 11 severe cases.

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11
Age (years)	61	46	62	48	75	88	62	62	55	60	82
Sex	Male	Male	Female	Male	Female	Male	Male	Male	Male	Female	Male
Occupation	Retired	Self- employed	Retired	Self-employed	Retired	Retired	Retired	Retired	Self- employed	Peasant	Retired
Comorbid conditions	Cerebral infarction; benign prostatic hyperplasia	No	Chronic obstructive pulmonary disease	Gastric ulcer	Hypertension; diabetes; hypothyroidism ; tricuspid valve replacement and tricuspid valvoplasty	No	Hypertension	Hypertension	Hyperlipidemia ; uarthritis	Hypertension ; diabetes	Gastric ulcer; tuberculosis

Onset of symptom	Cough; expectoration; chest stress; dyspnea; bloody sputum	Fever; cough; expectoration; chest stress; dyspnea	Fever; cough; expectoration; chest stress; dyspnea; bloody sputum	Fever; cough; expectoration; chest stress; dyspnea	Fever; cough; expectoration; chest stress; dyspnea; muscle ache	Fever; cough; expectoration; chest stress; dyspnea	Fever; cough; expectoration; chest stress; dyspnea	Fever; cough; expectoration; chest stress; dyspnea	Fever; cough; expectoration; chest stress; dyspnea	Fever; cough; expectoration; chest stress; dyspnea	Cough; expectoration; chest stress; dyspnea
Date of illness onset	2018.12.18	2019.01.08	2019.01.15	2019.01.15	2019.01.17	2019.01.20	2019.01.22	2019.02.01	2019.02.02	2019.02.02	2019.02.03
Date of admission	2019.01.15	2019.01.15	2019.01.18	2019.01.27	2019.01.17	2019.01.28	2019.01.29	2019.02.05	2019.02.02	2019.02.04	2019.02.05
Transferred to ICU*	2019.01.15	2019.01.15	2019.01.20	2019.01.27	2019.01.24	2019.01.28	2019.01.30	2019.02.05	2019.02.02	2019.02.04	2019.02.06
Date of specimen collected to be sequenced	2019.01.17	2019.01.15	2019.01.19	2019.01.28	2019.01.25	2019.01.29	2019.01.30	2019.02.07	2019.02.04	2019.02.05	2019.02.09
Spesis	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ARDS†	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	No
Acute RenalInjury	No	Yes	No	Yes	No	Yes	No	No	No	No	No
Secondary infections	No	Yes	No	No	No	No	No	No	No	No	No
Interval of illness onset to ARDS (days)	NA	7	5	12	NA	NA	8	4	1	1	NA
Interval of illness onset to sepsis (days)	NA	7	5	12	7	8	8	4	1	1	2
WBC§ ($\times 10^9$/L)	6.21	8.92	10.39	9.7	7.96	9.72	6.94	8.32	7.96	4.56	5.63
L (%)§	11.9	5.6	5.6	11.4	7.6	5.8	15.9	5	7.6	8.5	5.8
N (%)§	80.7	93.5	90.2	83.4	79.2	91.6	80.4	92.3	79.2	88.2	87.7
PT (s)§	17	10.2	11.3	14.5	42.6	28.1	12.1	11	11.3	13.2	11.5
APTT (s)§	46.5	32.1	43.3	33.1	60	54.3	49.6	37.6	34	41.9	34.9

PT-fbg (g/L)[§]	4.295	4.589	2.531	5.135	5.974	3.371	5.219	4.547	4.421	2.615	3.287
PCT (ng/ml)[§]	0.08	0.11	1.7	3.74	<0.05	3.31	1	0.76	0.08	1.95	<0.05
Sputum culture of G+/G-[‡]	(-)	*Stenotrophomonas maltophilia*	*Pseudomonas aeruginosa; Acinetobacter baumannii*	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Sputum culture of Fungal	*Candida albicans*	*Aspergillus fumigatus; Candida albicans*	NA	NA	(-)	NA	(-)	(-)	(-)	*Candida albicans*	(-)
Blood culture	NA	*E.casseliflavus ; S.xylosus*	NA	NA	NA	(-)	NA	(-)	(-)	(-)	(-)
Antiviral agent	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir
Interval of illness onset to antiviral therapy (days)	28	7	3	2	1	1	7	4	1	1	2
Duration of antiviral therapy (days)	12	18	13	2	6	9	14	13	2	13	4
Oxygen therapy	Nasal catheter	High flow oxygen	Mechanical ventilation	Mechanical ventilation	Mask	Nasal catheter	High flow oxygen	Mechanical ventilation	High flow oxygen	Mask	Nasal catheter
Antibiotic therapy	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Antifungal therapy	No	Yes	No	No	No	No	No	Yes	No	Yes	No
Glucocorticoid therapy	No	No	No	No	No	No	Yes	No	Yes	No	No
Intravenous immune globulin therapy	No	Yes	No	No	Yes	No	No	No	No	Yes	No
Length of stay in hospital (days)	12	32	14	5	5	5	21	12	2	17	4

Outcome	Improved to recovery	Died	Improved to recovery	Died	Improved to recovery	Died	Improved to recovery	Improved to recovery	Died	Improved to recovery	Improved to recovery
Date of death	NA	2019.02.16	NA	2019.01.28	NA	2019.02.04	NA	NA	2019.02.04	NA	NA

Table S7. Univariate analysis of risk factors for Human H9N2 severe cases with the mild cases as control.

Variables	Mild (n=5)	Severe (n=11)	*P* value
Age (≥65 years)	2 (40.0)	3 (27.3)	1.000
Sex (Male)	2 (40.0)	8 (72.7)	0.299
Underlying health condition	0	9 (81.8)	0.005
dyspea	1 (20.0)	11 (100)	0.003
chest stress	2 (40.0)	11 (100)	0.018
ARDS*	0	7 (63.6)	0.034
Sepsis	0	10 (90.9)	0.001
Interval of illness onset to antiviral therapy >3 days	1 (20.0)	4 (36.4)	0.622
Sputum cultureof G+/G-†	0	2 (18.2)	0.542
WBC§<4.0 × 10^9/L or WBC >1.0×10^{10}/L	1 (20.0)	1 (9.1)	1.000

*ARDS: acute respiratory distress syndrome

†Sputum culture of G+/G: culture and identify Gram-positive/Gram-negative bacterica in sputum

§ WBC: white blood cell count

Table S8. Univariate analysis of predictor for death of H9N2 human severe cases with who had recovered from illness as control.

Variables	**Death** (n=4)	**Recovery** (n=7)	**P value**
Age (≥65 years)	1 (25.0)	2 (28.6)	1.000
Sex (Male)	4 (100)	4 (57.1)	0.236
ARDS*	3 (75.0)	4 (57.1)	1.000
Spesis	4 (100)	6 (85.7)	1.000
Sputum cultureof G+/G-†	1 (25.0)	1 (14.3)	1.000
Interval of illness onset to antiviral therapy > 6 days	1 (25.0)	2 (28.6)	1.000
Acute Renal Injury	3 (75.0)	0	0.024
Secondary infections	1 (75.0)	0	0.364
Underlying health condition	2 (50.0)	7 (100)	0.109

*ARDS: acute respiratory distress syndrome

†Sputum culture of G+/G: culture and identify Gram-positive/Gram-negative bacterica in sputum