Original Article

Iron status is linked to disease severity after avian influenza virus H7N9 infection

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Background and Objectives: The high mortality rate of H7N9 strain of avian influenza virus (AIV) infected patients has been a major clinical concern. Iron overload increases the susceptibility of host for several kinds of microbial infection. However, the study on patients' iron and ferritin status associated with clinical outcome of AIV-H7N9 virus infection is poorly understood, and in order to explain the linkage we carried out this study. **Methods and Study Design:** We retrospectively collected serum from 46 patients infected with H7N9 virus from the hospital in Hangzhou city, Zhejiang province of China in 2013. We measured the level of serum iron and ferritin by Enzyme-Linked Immunosorbent Assay (ELISA). The correlation analysis of iron and ferritin with disease severity was done by SPSS 16.0 and MedCalc Software. **Results:** After H7N9 infection, there is a reduction in iron level and an increase in ferritin, hepcidin and C-reactive protein (CRP) level in patient's serum compared to those of the control (p<0.001), and there's little correlation between procalcitonin (PCT) level and H7N9 infection. At week 1 and week 2 post-infection, serum iron level is much lower and ferritin level is much higher in the patients who died later than those in the patients who survived. The sensitivity, specificity, and Area Under the Curve (AUC) of the assay was calculated with MedCalc software and they were 85.5%, 65.9% and 0.803 for iron and 84.9%, 80.7% and 0.900 for ferritin, 95.2%, 51.1% and 0.684 for PCT and 100%, 94.6% and 0.988 for CRP, respectively. **Conclusions:** Our study found that low serum iron and high serum ferritin levels are correlated with the disease severity of H7N9-infected patients and can predict fatal outcomes.

Key Words: H7N9, influenza, iron, ferritin, outcomes

INTRODUCTION

The avian influenza H7N9 outbreak in 2013 and its subsequent waves posed a great challenge to public health because of the high rates of morbidity and mortality.[1] Patients infected with AIV, are commonly characterized by fever and cough and rapidly developed pneumonia, and acute respiratory distress syndrome.[2] According to World Health Organization (WHO) report in 2019, H7N9 viruses have caused six seasonal epidemic waves in China with 1568 H7N9-infected cases and a fatality rate of about 40%,[3] although after adopting effective control methods there is only one case of human infection in the sixth wave, the risk profile has not changed. Several cases of limited human-human transmission of H7N9 have been reported, but recent sequence analysis of the AIV H7N9 virus indicates that it has evolved a more highly pathogenic H7N9 variant for both poultry and mammalian hosts, thereby increasing the potential public health threat.[4,5]

Current evidences have shown that besides CRP, hypercytokinemia factors, and some clinical parameters such as the PaO_2/FiO_2 ratio,[6-8] the angiotensin II,[9] certain cytokines and chemokine,[10] neutrophil-to-lymphocyte ratio (NLR),[11] have been suggested as prognostic biomarkers for the outcome of AIV-H7N9 infection. Secondary bacterial infections or coinfection occur from time to time during H7N9 disease.[12] PCT the same as CRP is a well-known acute phase response protein during pathogenic microbe infection, based on meta-analysis. Simon et al (2004) found that PCT is the diagnostic markers for bacterial infections.[13]

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Iron is an essential nutrient for maintaining health in all organisms with many biologic functions, including oxygen transport and cellular respiration.[14] Iron deficiency and overload both are harmful to cells and tissues, and leading to disease.[15] Iron is an indispensable micronutrient involved in various metabolic processes of cells. During microbial infection, pathogens acquire iron from their hosts by various means, while hosts try to prevent this.[16-18] Therefore, iron becomes the object of competition between the host and pathogen. The alteration of iron balance is associated with the severity of several infectious diseases, including malaria,[19] tuberculosis,[20] norovirus,[21] dengue virus,[22] Hepatitis B virus,[23] West Nile virus,[24] Hepatitis C virus,[25] bovine leukaemia virus[26] and Human Immunodeficiency virus type 127 infection. Iron balance is tightly regulated to meet the body's iron requirements and to avoid the toxicity associated with iron overload.[28,29] Iron is stored in the form of ferritin. Serum ferritin is a useful indicator for estimating body iron stores and potentially acts as a marker for inflammatory conditions.[26] Hepcidin is an important regulator that controls the iron absorption and storage.[30] The level of hepcidin and ferritin help to understand the iron status of individuals. However, the study on patients' iron status and its correlation with H7N9 virus infection hasn't been reported. Therefore, we investigated the iron status in H7N9-infected patients as well as in the human population without AIV infection.

METHODS

Human serum samples

Serum samples from a total of 46 AIV-H7N9 infected patients in 2013 with acute respiratory distress syndrome and multiorgan dysfunctions symptom were collected from the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China). Sera from same age and gender group of 83 healthy volunteers who were considered at high risk for H7N9 infection, as they shared the same life style of buying chickens in the live poultry market, were collected in ZiJinGang Campus Hospital of Zhejiang University during the same period as controls. Clinical information was collected by attending physicians on the same day of serum sampling. Other information about patients were either retrieved from their medical records or acquired directly from them via a questionnaire. H7N9 virus infection was confirmed by *in vitro* virus culture and/or reverse-transcription polymerase chain reaction (RT-PCR) analysis of test samples. Serum antibodies against the H7N9 virus was determined by using a hemagglutination inhibition assay with live virus and chicken red blood cells in a biosafety level 3 laboratories according to the protocol developed by the Chinese Centre for Disease Control and Prevention. All specimens were stored at -80 ℃ until further analysis.

Experiments using human samples were approved by the Institutional Review Board of the First Affiliated Hospital, College of Medicine, Zhejiang University (reference number 2013-131) and conducted in accordance with the principles expressed in the Declaration of Zhejiang University. Written informed consent was obtained from all adult subjects.

ELISA

Serum iron was measured with the Iron/TIBC Reagent Kit according to the manufacturer's instructions (Pointe Scientific, Inc). Serum ferritin and hepcidin concentrations were measured with the FERR4 Tina-quant Ferritin Gen 4.0 kit (Roche, Basel, Switzerland) and the Intrinsic Hepcidin IDxTM ELISA Kit (Intrinsic Lifesciences, CatLog No. ICE-007). Procalcitonin concentrations was measured with the Ancillary Reagent Kit 2 (5 plates) (R&D Systems, CatLog No. DY008), and CRP concentrations was measured with the commercial kit (R&D Systems, CatLog No. DCRP00).

Statistical analysis

The Mann-Whitney U test or chi-squared-test was performed to determine the differences between infected and control groups. Wilcoxon matched-pair test was employed to assess the difference during the first, second and the third week of disease onset among the same patients. We calculated receiver operating characteristic curves for predictive analysis. All statistical tests were performed with SPSS 16.0 for Windows (SPSS, Inc.). Receiver operating characteristic (ROC) curves calculation was performed with SPSS 16.0 for Windows and MedCalc Software. A p-value <0.05 was considered statistically significant.

RESULTS

Patients characteristics

The basic clinical information and treatments are shown in Table 1. The mean age of 46 inpatients was 60 years range from 37 to 84, and 58.7% (27 patients) were male. Of 46 inpatients, 11 (23.9%) patients had a history of smoking, and 34 patients had one or more other kind of symptoms such as hypertension, diabetes, and coronary heart disease. Our previous study showed that the antibodies of the patients fourteen days after symptom onset, elevated levels in 65.8% of patients who survived but in only 28.6% of those who died.31

Serum iron status may reflect disease outcomes in H7N9-infected patients

To categorize the samples on the basis of time points, we collected the sera at three different time intervals: first group was collected within the first 7 days of disease onset (n=13), another was collected between 8- and 14-days (n=18), and last one was between 14-21 days after disease onset (n=15) (Figure 1A). Intriguingly, at all 3 stages, serum iron levels were markedly lower in infected individuals compared to the uninfected healthy controls (Figure 1A). On the contrary, there was a marked elevation of serum ferritin levels at all stages (Figure 1A). Apart from iron and ferritin, we also measured the serum hepcidin, a kind of iron homeostasis regulator whose expression affects the iron status. The results showed that in addition to ferritin, hepcidin and CRP levels were also significantly elevated (Figure 1B). Based on these results we postulate that abnormal iron status involving inflammatory response during virus infection, contributes to influenza disease pathogenesis.

To investigate changes of iron and ferritin levels during disease progression of H7N9-infected patients, we ana-

Table 1. Clinical characteristics of the 46 patients with H7N9 virus infection

Characteristics	Value of patient	Value of control
Male sex-no. (%)	27 (58.7%)	42 (50.6%)
Age		
Year (Median)	37-84 (60)	45-65 (55)
Subgroup-no. (%)		
>65	14 (30.4%)	20 (24.1%)
<65	32 (69.6%)	63 (75.9)
Pneumonia-no. (%)	46 (100%)	0 (0%)
H7N9 positive day (Median)	7-11 (8)	0
Titer of H7N9 antibody (HI)	≥25	<21
Coexisting conditions-no. (%)		
Current smoker-no. (%)	11 (23.9%)	12 (14.5%)
Hypertension	18 (39.1%)	9 (10.8%)
Diabetes	6 (13%)	3 (3.6%)
Chronic obstructive pulmonary disease	0 (0%)	0 (0%)
Coronary heart disease	6 (13%)	0 (0%)
Chronic liver disease	0 (0%)	0 (0%)
Chronic renal disease	0 (0%)	0 (0%)
Cancer	2 (4.3%)	0 (0%)
Immunosuppression	2 (4.3%)	0 (0%)
Complication-no. (%)		
Second infection	12 (26.1%)	0 (0%)
Bacterial and fungal	5 (10.9%)	0 (0%)
Bacterial	10 (21.7%)	0 (0%)
Fungal	7 (15.2%)	0 (0%)
Treatment-no. (%)		
Administration of oseltamivir or peramivir	46 (100%)	0 (0%)
Antibiotic therapy	17 (37%)	0 (0%)
Glucocorticoid therapy	9 (19.6%)	0 (0%)
Mechanical ventilation	4 (8.7%)	0 (0%)
Extracorporeal membrane oxygenation	2 (4.3%)	0 (0%)
Artificial liver support system	6 (13%)	0 (0%)
Clinical outcome		
Death-no. (%)	4 (8.7%)	0 (0%)
Mean of days from onset of symptoms to death	26	-
Discharge from hospital-no. (%)	42 (91.3%)	-
Length of stay in hospital-day (Median)	12-37 (19)	-
WBC (10^9/L)-no.(Median)	1.7-12.9 (4.1)	-
LY (10^9/L)-no.(Median)	0.1-1.3 (0.6)	-

†HI: hemagglutination inhibition; WBC: white blood cell; LY: lymph.

lysed serum iron and ferritin levels in H7N9-infected patients at the first and second week after the infection (Figure 2). Total 19 H7N9-infected patients were divided into three groups according to the disease outcome: patients who were discharged from the hospital within 28 days, those who were discharged after 28 days and those who died (Figure 2). Interestingly, the level of serum iron/ferritin had no significant differences between the first and the second week groups compared to those who were discharged within 28 days or more or died (Figure 2). However, in the group of patients who died, serum iron levels remained low and ferritin levels remained high during the second week of illness (Figure 2). Therefore, serum iron status in H7N9-infected patients may correspond to the disease severity and may be predictive of the disease outcome.

Serum iron/ferritin levels can predict fatal outcomes

We further analysed whether iron levels were linked to fatal outcomes. Indeed, serum iron levels during the first week of H7N9 illness were significantly lower in the group of patients who died than the group who were discharged from the hospital within 28 days, and serum iron levels during the second week of H7N9 illness were significantly lower in the group of patients who died than the group who were discharged from the hospital within 28 days or more than 28 days (Figure 3). Moreover, the serum ferritin levels were significantly increased accordingly.

To further analyse the association between iron and ferritin dynamics and the severity of the disease symptoms, we calculated the predictive value of fatal outcome using SPSS software. The AUC of free iron and ferritin levels among H7N9 virus-infected patients was 0.803 and 0.900, respectively (Figure 4, and Supplementary table 1). Furthermore, we also evaluated other biomarkers for infectious disease, such as PCT which was indicative to bacterial infection[32] with AUC of 0.684 and inflammation biomarker CRP[33] with AUC of 0.988 (Figure 4, and Supplementary table 1). We also used MedCalc software and got the similar results to ROC (Supplementary table 2-5). The sensitivity and specificity of this analysis calculated with MedCalc software were 85.5% and 65.9% for iron concentration, 84.9% and 80.7% for ferritin level, 95.2% and 51.1% for PCT level and 100% and 94.6% for CRP respectively (Supplementary tables 2-5). In addition, the positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio within

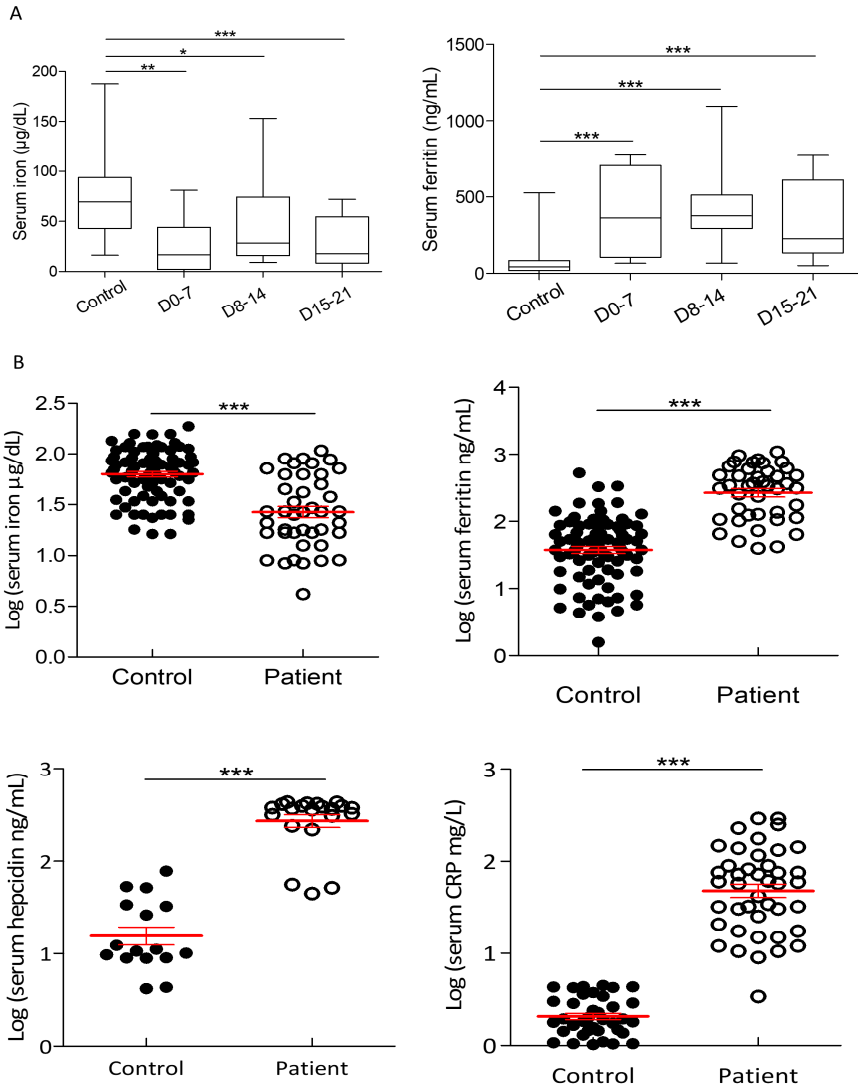

Figure 1. Iron and ferritin levels in the serum of H7N9-infected patients. (A) Serum iron and ferritin levels in patient with H7N9 infection (n=46) and healthy controls (n=83). The sample sizes for each group were: day 0-7 group n=13, day 8–14 group n=18 and day 15 group n=15. (B) Serum iron, ferritin, hepcidin and CRP levels in patients with the whole H7N9 infection process. All experiments were in duplicate. The horizontal lines represent the mean value of each group. Mann–Whiney U test and Wilcoxon matched-pair test were used in the statistics. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

95% confidence intervals were also determined (Supplementary table 2-5).

Taken together, these data suggested that the reduction in iron level and the increase in ferritin level in the serum from H7N9 virus-infected patients were correlated with the severity of influenza virus infection.

DISCUSSION

In this study, we report for the first time that the level of serum iron in patients with AIV-H7N9 infection may potentially predict the patient mortality, although the specificity of the patients' serum iron level was only 65.9%, which was not suitable for the diagnosis of influenza virus infection. Consistent with our findings, previous studies have reported lower iron and higher ferritin levels in patients suffering from several infectious diseases such as norovirus[21] and malaria.[19] In addition, similar results appeared in patients with chronic alcoholic liver disease.[34] We infer that the plausible cause of this phenomenon is inflammation.

Previous studies have described the relationship among infection, inflammatory response and iron metabolism.[35,36] This is well known that microorganism infection causes inflammation which alters the iron metabolism.[21] CRP is not only an infection and inflammatory marker, but also a regulator of inflammatory factors.[37] Previous studies have found that high level of CRP is correlated with a high fatality rate in AIV-H7N9-infected patients.[11,38,39] In this study, we also observed the higher CRP levels in H7N9 infected patient compared to the uninfected normal subjects. High hepcidin level in patients suggests that there may be some correlation between hepcidin and the severity of H7N9 infection. Hepcidin is an important regulator that regulates many processes of metabolism, e.g. iron absorption and its turnover. Studies had showed that hepcidin and ferritin levels are both regulated by iron status and the inflammation caused by infection, so as to maintain the balance of iron metabolism.[40-42] Hepcidin level is regulated by Ft-H (encoding ferritin heavy chain) and other iron metabolism genes.[40] We propose that H7N9 influenza virus infection leads to

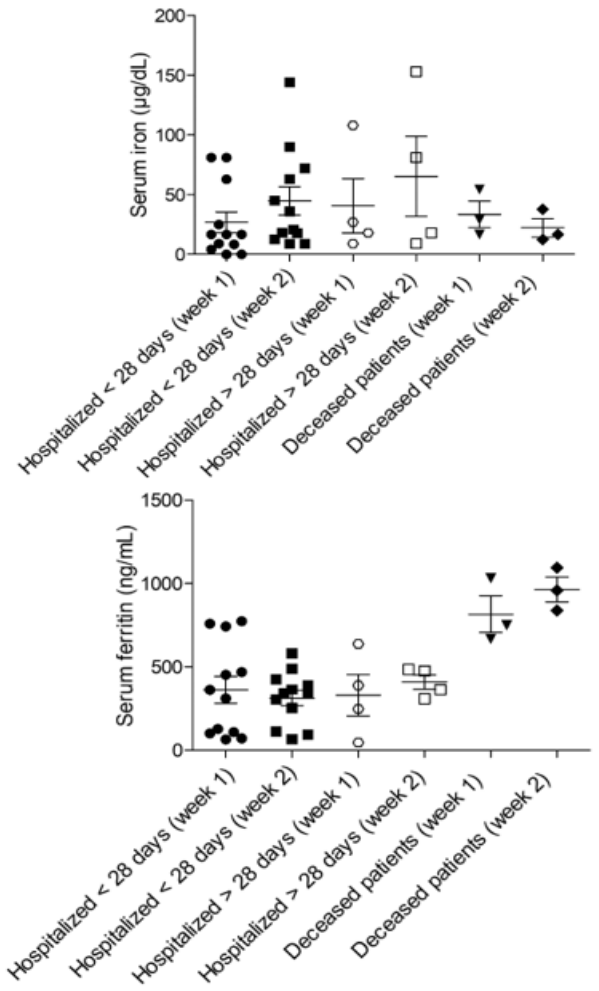

Figure 2. Serum iron status may reflect disease outcomes in H7N9-infected patient. Serum iron and ferritin levels at week 1 and week 2 in H7N9-infected patients hospitalized less than 28 days (n=12), hospitalized longer than 28 days (n=4) and deceased (n=3). All experiments were in duplicate. The horizontal lines represent the mean value in each group. Mann–Whiney U test and Wilcoxon matched-pair test were used in the statistics.

inflammation which changed the hepcidin and ferritin level and reduced the serum iron, to enhance the resistance of humans against the virus infection.

PCT is a common biomarker of bacterial infection.[32] Our results show that there's little correlation between PCT and the diagnosis of patients infected with H7N9 influenza. And the level of PCT (<10 pg/mL) is much lower than clinical reference (100 pg/mL) (supplemental table 6) in both control and patients. On the one hand, these results indicated that the patients did not have secondary bacterial infection, and on the other hand, it also confirms that low serum iron and high ferritin levels were caused by H7N9 influenza virus infection. All the above results suggest that the H7N9 infection causes severe inflammatory response and hyperferritinemia.

Our current findings are in agreement with the study mentioned the inflammation by H7N9 infection.[2,10] The inflammation is associated with clinical outcome in H7N9 infected patients. In previous study, The NLR in the fatal group was significantly higher than that in the survival group and was independently associated with fatality.[11] Low proportions of T cells,[43] pronounced lymphopenia and high chemokine and cytokine levels[44] were observed in H7N9-infected patients, particularly in those patients whose disease outcome was fatal later on. The presence of antibodies improves clinical outcome in infected patients.[31,45] The proinflammatory cytokine response, induced by a combined Th1/Th17 cytokine, are partially responsible for the disease progression in patients infected with H7N9 influenza virus.[10] In addition, there are several other factors associated with fatality during H7N9 virus infection, including age, time from illness onset to antiviral therapy initiation, and secondary infection.[3] In this study, we investigated the clinical features and potential complications in H7N9-infected patients. The knowledge about the clinical features, potential complications and other specific factors in the current study might be helpful in evaluating disease severity and designing better therapeutic measures.

Conclusion

Our study demonstrates that there is a clear correlation between patients' iron status and disease severity, and that low iron level and high ferritin level can serve as the potential biomarker for fatal outcomes and disease progression in the AIV-H7N9 influenza virus infected individuals.

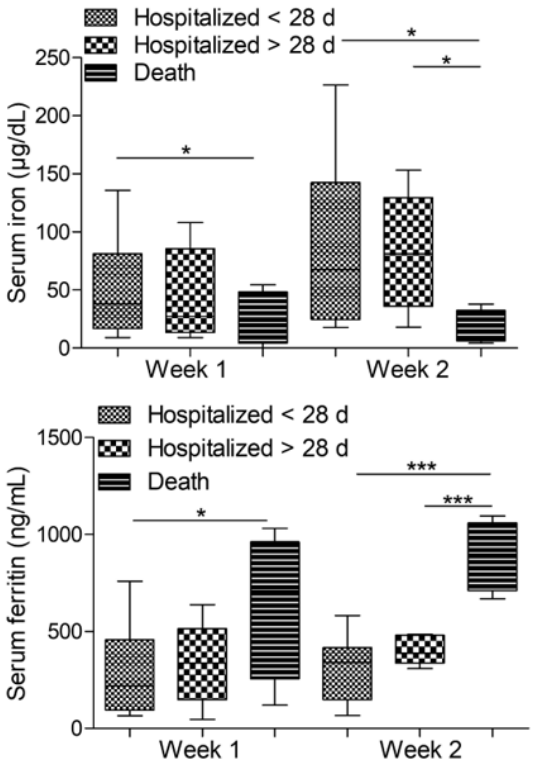

Figure 3. Fatal outcome is linked to iron status in H7N9-infected patients. Serum iron and ferritin concentrations from the first and second week of the patients infected with H7N9 virus in different outcome groups. The number of patients and the time-period in which they died are as follows. Patients with serum collected during the first week of illness: patients hospitalized less than 28 days (n = 10), hospitalized longer than 28 days (n=5) and death (n=4). Patients with serum harvested during the second week of illness: patients hospitalized less than 28 days (n=12), hospitalized longer than 28 days (n=5) and death (n=4). The horizontal lines represent the mean value in each group. Mann–Whiney U test was used in the statistics. $^{*}p<0.05$, $^{***}p<0.001$.

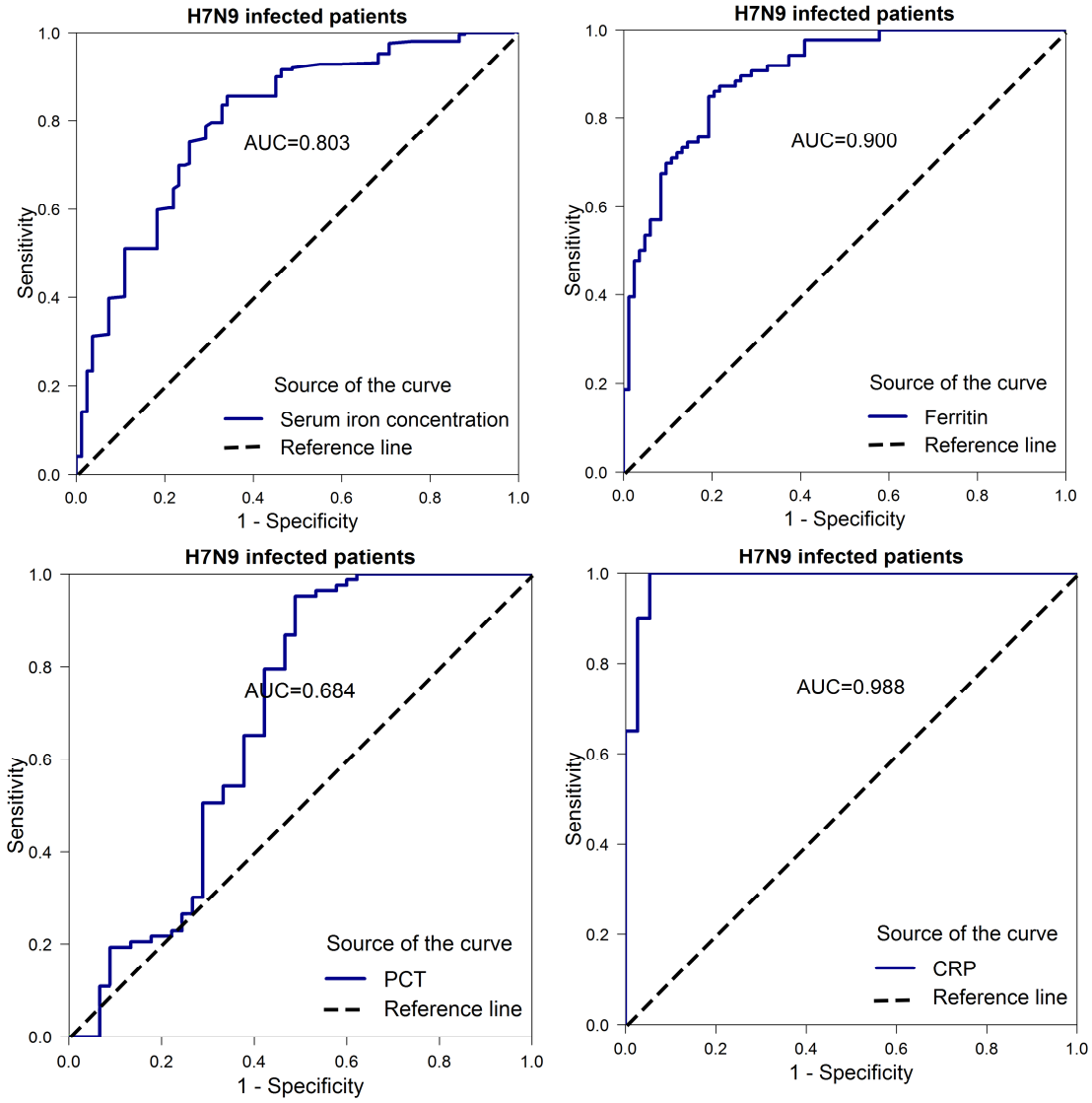

Figure 4. ROC curve of serum iron/ferritin levels and other biomarkers. ROC curves of the serum iron, ferritin, PCT, and CRP levels with the AUC of 0.803, 0. 900, 0.684 and 0.988 separately during H7N9 infection are shown.

AUTHOR DISCLOSURES

The authors report no conflicts of interest.

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Supplementary table 1. ROC curve of characteristics in H7N9 virus infected illness onset

Characteristic	Area under the ROC curve	Std. Error	*p* value	95% Confidence Interval	
				Lower bound	Upper bound
Serum iron concentration	0.803	0.029	1.81E-16	0.747	0.859
CRP	0.998	0.01	2.59E-17	0.968	1
PCT	0.684	0.057	0.000601	0.573	0.796
Ferritin	0.900	0.023	3.00E-19	0.855	0.944

CRP: C-reactive protein; PCT: procalcitonin.

Supplementary table 2. ROC curve of serum iron levels during H7N9 virus infection

Area under the ROC curve	0.803
Standard Error†	0.0288
95% Confidence interval‡	0.747-0.860
z statistic	10.519
Significance level *p* (Area=0.5)	<0.0001
Youden index J§	0.5140
Associated criterion	>29.32961
Sensitivity	85.54
95% Confidence interval	80.6-89.7
Specificity	65.85
95% Confidence interval	54.6-76.0
PPV	88.4
95% Confidence interval	83.6-92.1
NPV 94.4	60
95% Confidence interval	49.1-70.2
PLR	2.51
95% Confidence interval	1.8-3.4
NLR	0.22
95% Confidence interval	0.2-0.3

PPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio; NLR: negative likelihood ratio.
†DeLong et al., 1988 (number the reference according to the sequence it appears in text)
‡AUC±1.96 SE
§Taking into account disease prevalence (75.2%) and estimated costs: cost False Positive: 1; cost False Negative: 1; cost True Positive: 0; cost True Negative: 0.

Supplementary table 3. ROC curve of serum ferritin levels during H7N9 virus infection

Area under the ROC curve	0.900
Standard Error†	0.0229
95% Confidence interval‡	0.855-0.944
z statistic	17.439
Significance level P (Area=0.5)	<0.0001
Youden index J§	0.6561
Associated criterion	≤92.1668752
Sensitivity	84.88
95% Confidence interval	75.5-91.7
Specificity	80.72
95% Confidence interval	70.6-88.6
PPV	82
95% Confidence interval	72.5-89.4
NPV 94.4	83.7
95% Confidence interval	73.8-91.1
PLR	4.4
95% Confidence interval	2.8-6.9
NLR	0.19
95% Confidence interval	0.1-0.3

PPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio; NLR: negative likelihood ratio.
†DeLong et al., 1988 (number the reference according to the sequence it appears in text)
‡AUC ± 1.96 SE
§Taking into account disease prevalence (75.2%) and estimated costs: cost False Positive: 1; cost False Negative: 1; cost True Positive: 0; cost True Negative: 0

Supplementary table 4. ROC curve of serum PCT levels during H7N9 virus infection

Area under the ROC curve	0.684
Standard Error†	0.0573
95% Confidence interval‡	0.572-0.796
z statistic	3.21
Significance level P (Area = 0.5)	0.0013
Youden index J§	0.4629
Associated criterion	>0.16
Sensitivity	95.18
95% Confidence interval	88.1-98.7
Specificity	51.11
95% Confidence interval	35.8-66.3
PPV	78.2
95% Confidence interval	68.9-85.8
NPV 94.4	85.2
95% Confidence interval	66.3-95.8
PLR	1.95
95% Confidence interval	1.4-2.6
NLR	0.094
95% Confidence interval	0.03-0.3

PPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio; NLR: negative likelihood ratio.
†DeLong et al., 1988 (number the reference according to the sequence it appears in text)
‡AUC ± 1.96 SE
§Taking into account disease prevalence (75.2%) and estimated costs: cost False Positive: 1; cost False Negative: 1; cost True Positive: 0; cost True Negative: 0

Supplementary table 5. ROC curve of serum CRP levels during H7N9 virus infection

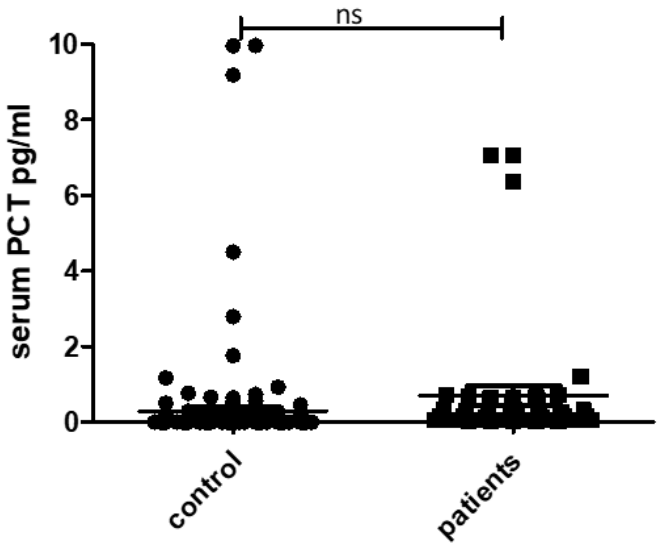
Area under the ROC curve	0.988
Standard Error†	0.00998
95% Confidence interval‡	0.968-1.000
z statistic	48.905
Significance level P (Area = 0.5)	<0.0001
Youden index J§	0.9459
Associated criterion	≤4.29
Sensitivity	100.00
95% Confidence interval	95.5-100.0
Specificity	94.59
95% Confidence interval	81.8-99.3
PPV	97.6
95% Confidence interval	91.5-99.7
NPV 94.4	100
95% Confidence interval	90.0-100.0
PLR	18.5
95% Confidence interval	4.8-71.2
NLR	0
95% Confidence interval	NULL

PPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio; NLR: negative likelihood ratio
†DeLong et al., 1988 (number the reference according to the sequence it appears in text)
‡AUC ± 1.96 SE
§Taking into account disease prevalence (75.2%) and estimated costs: cost False Positive: 1; cost False Negative: 1; cost True Positive: 0; cost True Negative: 0

Supplementary table 6. Clinical Reference

Area under the ROC curve	0.988
Standard Error[†]	0.00998
95% Confidence interval[‡]	0.968-1.000
z statistic	48.905
Significance level P (Area = 0.5)	<0.0001
Youden index J[§]	0.9459
Associated criterion	≤4.29
Sensitivity	100.00
95% Confidence interval	95.5-100.0
Specificity	94.59
95% Confidence interval	81.8-99.3
PPV	97.6
95% Confidence interval	91.5-99.7
NPV 94.4	100
95% Confidence interval	90.0-100.0
PLR	18.5
95% Confidence interval	4.8-71.2
NLR	0
95% Confidence interval	NULL

PPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio; NLR: negative likelihood ratio.
[†]DeLong et al., 1988 (number the reference according to the sequence it appears in text)
[‡]AUC ± 1.96 SE
[§]Taking into account disease prevalence (75.2%) and estimated costs: cost False Positive: 1; cost False Negative: 1; cost True Positive: 0; cost True Negative: 0



Supplementary figure 1. Procalcitonin (PCT) level in serum of patients and control. Serum PCT level in patients with the whole H7N9 infection process. All experiments were in duplicate. The horizontal lines represent the mean value of each group. Mann–Whiney U test and Wilcoxon matched-pair test were used in the statistics. $^{*}p<0.05$.