Proteomic Analysis of Avian Influenza A (H7N9) Patients within a Family Cluster

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Abstract

**Background:** To date, there is limited information on the progression of human infections of avian influenza virus A (H7N9). This study investigated differential blood protein profiling of an H7N9-infected family cluster to find a slice of crucial proteins concerning disease attack and virus clearance.

**Materials and Methods:** Plasma samples from one family cluster (including one index case and one asymptomatic case) were collected at four time points. The protein profiles were identified by isobaric tagging for relative and absolute quantification-based quantitative differential LC/MS/MS, and their functional annotations were analyzed by PANTHER and STRING tools.

**Results:** A total of 1257 nonredundant proteins were identified from 3027 unique peptides. Three differential protein profiles for each subject were generated by comparing relative protein abundance between samples of each of the first three time points and the last time point.

**Conclusions:** Significant perturbation of the response upon viral infection occurred immediately after confirmation of H7N9 virus infection. The differential protein profiles shed further light on distinguishing the index case from the asymptomatic one. Furthermore, apolipoproteins may play an important role in the progression of the disease.

Keywords: Asymptomatic infection, avian influenza A (H7N9) virus, plasma proteome

Introduction

On March 31, 2013, three human infections with a novel avian influenza A (H7N9) virus were identified in Shanghai and Anhui Province in Southeastern China. As of February 28, 2014, a total of 375 laboratory-confirmed H7N9 infections and 115 deaths have been reported; 367 of those cases were from Mainland China.\(^{[1]}\) Unlike other influenza A (H7) infections, H7N9 cases usually present with lower respiratory tract disease symptoms and progress to acute respiratory disease syndrome and multiple organ failure, ultimately resulting in death.

On April 12, 2013, a family cluster infected with the H7N9 virus was found in Beijing, China. One patient presented with pneumonia while the other patient was asymptomatic. The different clinical features between these two and previous cases indicated that the H7N9 virus can trigger a wide spectrum of symptoms in humans ranging from none to severe.

Although poultry exposure was associated with H7N9 infection, the transmission route to human remains unclear. In addition, the pathogenic mechanism still needs to be elucidated. Previous research showed that the H7N9 virus can bind to human-type (α2, 6-linked sialic acid) receptors and invade epithelial cells in the lower respiratory tract and type II pneumocytes in the alveoli, where the virus can replicate efficiently.\(^{[2,3]}\) The established H7N9 infection then triggers acute cytokine, chemokine, and antibody responses, resulting in fever and disease persistence.\(^{[2,4]}\)

So far, no proteomic studies of H7N9 infections have been reported. Previous studies using cytometric bead arrays have...
shown high levels of a series of cytokines and chemokines in the acute sera from H7N9 patients.\(^2\)\(^3\)\(^4\) In addition, elevated concentration of cytokines in the plasma (hypercytokinemia) was indicative of poor prognosis in patients with H7N9 infections.

Moreover, no preexisting immunity was detected in serum samples of H7N9 patients of any age group, and no detectable cross-reactive antibodies against the H7N9 virus were present in any age group after current seasonal vaccination, demonstrating that the human population is naïve to the H7N9 virus.\(^2\) Consequently, in addition to immunologic proteins with known functions, many proteins with unknown functions will be found in the blood in the process of defending the body against the H7N9 virus invasion, which may contribute to the wide range of symptoms seen in patients infected with H7N9 virus.

Therefore, to better understand the mechanism of defense against H7N9 infection, we analyzed the quantitative protein profiling of sequential plasma samples from two H7N9-infected patients in the family cluster found in Beijing, using isobaric tagging for relative and absolute quantification (iTRAQ)-based quantitative differential LC/MS/MS. Given the limited information on the disease progression after H7N9 virus infection, this study may be useful in finding important proteins related to disease cure and virus clearance.

**Materials and Methods**

**Epidemiological investigation and sample collection**

The index case, a 7-year-old girl from a family of chicken retailers in Beijing, was diagnosed with H7N9 virus-associated pneumonia on April 12, 2013. Epidemiological investigation and potential-infected patients screening were conducted immediately after the first case was confirmed.

Among the family contacts of the patient, only the index patient’s mother, a 33-year-old woman, was identified to be asymptptomatically infected with the H7N9 virus.

Plasma samples of both patients were collected at four time points, at the time of diagnosis (2 days post first fever for the index patient while for the second case was 1 day after the index case diagnosed), 1 later, 2 later, and 4 weeks later for further iTRAQ analysis.

**Ethical statement**

Ethical approval was obtained from the Ethics Committee of the Beijing Center for Disease Control and Prevention. Sample collection in this study was agreed by either the patient or the patient’s guardian as appropriate with prior informed consent.

**High-abundance protein depletion and peptide labeling by isobaric tagging for relative and absolute quantification**

Serum samples were centrifuged at 10,000 g for 10 min to sediment the precipitates. High-abundance proteins were depleted from 250 µl of each sample using a ProteoMiner protein enrichment kit (Bio-Rad, Hercules, California, USA.) according to manufacturer’s instructions. The process yielded 60 µl of depleted fraction per sample. The depleted samples were reduced and alkylated by 10 mM dithiothreitol and 55 mM iodoacetamide. The treated proteins were precipitated in 80% acetone at −20°C overnight, and the precipitants were resuspended in 0.8 M urea and 500 mM tetraethylammonium bicarbonate, pH 8.5. The protein concentrations were determined using the Bradford method followed by a 16 h trypsin digestion at 37°C. The tryptic peptides were labeled by the 8-plex iTRAQ reagents (AB Sciex, Foster City, CA, USA) following the manufacturer’s protocol. After 2 h of labeling reactions, the reaction solvents were removed by speed-vacuum, and the labeled peptides were dissolved in 20 mM NH4 FA, pH 10, for the following experiments.

**Peptide fractionation by strong cation exchange**

The labeled peptides were loaded onto a strong cation exchange (SCX) column (Luna SCX, 4.6 mm × 250 mm, Phenomenex, CA, USA) and eluted by a step linear elution program, 0–10 min equilibrated in buffer A (25% acetonitrile [ACN], 20 mM KCl, and 10 mM KH₂PO₄, pH 3.0), 10–15 min fast elution from 0% to 5% of buffer B (25% ACN, 1 M KCl, and 10 mM KH₂PO₄, pH 3.0), 15–50 min linear elution from 5% to 30% of solution B, and 50–55 min washing elution from 30% to 80% of solution B. The high-performance liquid chromatography (HPLC) procedures were performed in a Prominence HPLC system (Shimadzu, Nakagyo-ku, Kyoto, Japan) with a flow rate of 1.0 ml/min, and the peptides were monitored at 214 nm. The fractioned peptides were collected at one tube/min during the linear elution period.

**Peptide identification by nano-reversed phase high-performance liquid chromatography and mass spectrometry**

The peptides were first identified by MALDI-TOF/TOF MS (Bruker Daltonics, Billerica, MA), and the fractions were further pooled to average the peptide content. The eluted fractions were transferred to a nano-RP column (5 mm Hypersil C18, 100 mm × 75 mm, Thermo Fisher Scientific, Waltham, MA, USA) mounted in a Prominence nano-HPLC system (Shimadzu, Nakagyo-ku, Kyoto, Japan) and were eluted with ACN gradient from 5% to 40% containing 0.1% formic acid, for 95 min at 400 nL/min. The elutions were directly entered into Q Exactive hybrid quadrupole-Orbitrap MS (Thermo Fisher Scientific), set in a positive ion mode and data-dependent manner with full MS scan from 350 to 6000 m/z, resolution at 70,000 MS/MS scan with higher collision energy dissociation mode, resolution at 17,500.

**Database searches for peptide and protein identification**

The raw MS/MS data were converted into MGF format by Proteome Discoverer 1.3 (Thermo Fisher Scientific, Waltham, MA, USA). The exported MGF files were searched by Mascot 2.2 (Matrix Science, Boston, MA) against the NCBI human database. Several parameters in Mascot were set for peptide searching including iTRAQ 8-plex for quantification, tolerance of one missed cleavage of trypsin, carbamidomethylation for
cysteine as fixed modification, and oxidation for methionine as variable modification. The precursor mass tolerance was 10 ppm and the production tolerance was 20 mmu.

**Quantitative data analysis for the isobaric tagging for relative and absolute quantification labeling peptides**

A unique protein with at least two unique peptides, with a false discovery rate (FDR) <0.01, was qualified for further quantification data analysis. The fold changes in protein abundance were defined as the median ratio of all significantly matched spectra with tag signals.

**Bioinformatic and statistical analysis**

Gene ontology (GO) analysis of the H7N9-related protein database was performed using PANTHER. STRING was utilized to search for protein–protein interaction networks. P < 0.05 was considered statistically significant.

**RESULTS**

**Establishment of H7N9 virus infection-related plasma protein database**

Using a Q Exactive MS with high resolution and fast MS/MS scan, we achieved satisfactory mass signals for plasma protein identification and quantification. In total, 284,696 MS/MS spectra were acquired from the technique duplicates, and the spectra annotation rates were approximately 20%. A total of 1257 proteins were identified from 3027 unique peptides (FDR < 0.01). The detailed information regarding the peptide or protein identification and quantification derived from Mascot and Proteome Discoverer 1.3 are available in Supplementary File 1.

The relative protein abundance of the first three time points was individually compared with the relative protein abundance of the last time point to generate three differential protein profiles for each subject. For the index case, the numbers of proteins identified in the plasma samples at 0-, 1-, 2-week after diagnosis through polymerase chain reaction (PCR) test were 289, 284, and 218, respectively, whereas for the second case, the number of proteins were 351, 214, and 255 for the time points.

The ratios above 2 and under 0.5 were considered as significant changes in protein abundance, and three differential protein profiles for each subject were generated as shown in Table 1 [Supplementary File 2]. The numbers of the differentially regulated proteins for the same patient were distinct across all three time points. For the index case, 201 proteins (71 upregulated and 134 downregulated), 158 proteins (97 upregulated and 61 downregulated), and 130 proteins (63 upregulated and 67 downregulated) were differentially expressed in the plasma samples at 0-, 1-, and 2-week after confirmation through PCR test, respectively. For the second case, the numbers of differential proteins were 207 (47 upregulated and 160 downregulated), 90 (41 upregulated and 49 downregulated), and 155 (25 upregulated and 130 downregulated) according to the three time points, respectively. At each postinfection stage, the differential protein profiles between the index case and the second case were quite distinct, with a few overlapping proteins. In addition, the second case, the asymptomatic one, presented more differential proteins in blood than the index case at 0- and 2-week after confirmation.

**Bioinformatic analysis of differential protein profiles of H7N9 infection cases**

To obtain a systematic overview of the protein profiles for each case at each time point, the proteins were first categorized according to their cellular component by GO analysis. With the PANTHER tool, extracellular proteins accounted for most of the total proteins in each plasma sample, as shown in Figure 1. Biological process categories were also enriched by the PANTHER tool. The GO terms were almost identical for the proteins of the two cases except the terms at the 1-week time point of the asymptomatic case. The categories were mainly about stimulus-related GO terms (i.e., “response to external stimulus,” “response to stimulus,” and “complement activation”), immunity-related GO terms (i.e., “immune system process” and “immune response”), cell adhesion-related GO terms (i.e., “cell-cell adhesion,” “cell adhesion,” and “biological adhesion”), blood coagulation, lipid transport, and proteolysis [Figure 2]. There were no significant categories at the 1-week time point of the asymptomatic case.

**Trend analysis of differential proteins during the postinfection period**

For each case, there were two groups of proteins with an upward or downward expressing change according to the postinfection time points [Supplementary File 3]. The protein levels in sequential plasma samples of the two subjects are shown separately in Figure 3. For the index case, 28 proteins showed an upward trend, whereas 16 proteins showed a downward trend. For the second case, the changing trend of 44 proteins was upward and the changing trend of 34 proteins was downward. As shown in Table 2, there were 18 proteins with an upward or downward change pattern in both cases. Among these proteins, ten proteins (include C4B precursor, HP, CFHR5, and APOC4) showed the same changing trend in both

| Table 1: Number of differentially expressed proteins in plasma samples at different time points after avian influenza A virus infection |
|---|---|---|---|---|
| **Subjects** | **Time points** | **Upregulated** | **Downregulated** | **In total** |
| The | 0-week | 71 | 134 | 201 |
| index case | 1-week | 97 | 61 | 158 |
| | 2-week | 63 | 67 | 130 |
| The second case | 0-week | 47 | 160 | 207 |
| | 1-week | 41 | 49 | 90 |
| | 2-week | 25 | 130 | 155 |

*0-week, 1-week, and 2-week successively represent the differential protein profiles in the samples collected at 0-week, 1-week, and 2-week after PCR test compared to that collected at 3-week after PCR test. PCR: Polymerase chain reaction
cases and eight proteins showed an opposite trend in these two cases (include A2M precursor, CD5L, TF precursor, and PZP).

The two groups of proteins with time-dependent expression pattern (i.e., an upward or downward tendency) for each case were input for the network analysis by STRING, to describe the protein–protein interactions. The core proteins in the network of the index case were APOC3, APOC4, APOC5, and CETP. In the network of the second case, the core proteins include APOC1, APOC2, HGFAC, and IGHG1. In addition, for the purpose to investigate the interaction of the different time-related proteins between the two subjects, another two networks were established. The core different proteins in the index case were APOC3, APOC5, and CETP whereas in the second case were APOC1, APOC2, HGFAC, and IGHG1. The core proteins involved in these four networks were mainly belonged to apolipoprotein family [Figure 4].

**Discussion**

The avian-specific genome and the antigenically distinct nature of H7N9 virus surface glycoproteins led to the absence of protective neutralizing antibodies for H7N9 virus in the
human population. Therefore, H7N9 virus remains a global public health concern because of its pandemic potential and its high human mortality rate. So far, a majority of the people infected with H7N9 virus had a critical illness, whereas the remainders were either mild cases or asymptomatic ones. Some studies conducted in the epidemic areas like Zhejiang Province showed that the levels of cytokines and chemokines in the plasma samples of patients infected with H7N9 were increased, for example, IL-6 and IP-10 were significantly elevated in severe H7N9 patients compared to nonsevere ones, suggesting

Figure 2: Analysis of the major biological processes using the PANTHER tool. The biological processes were significantly enriched with the Bonferroni correction for multiple testing and the list of NCBI Homo sapiens genes as the reference list. The biological processes and corresponding significances (negative log of the P value) are shown on the X- and Y-axis, respectively. (a-c) The results of the index case at 0-, 1-, and 2-week time point, respectively. (d-f) The results of the second case at 0-, 1-, and 2-week time point, respectively.
that the pro-inflammatory cytokine responses are partially responsible for the disease progression after H7N9 virus infection. However, the change of cytokines and chemokines is just the tip of the iceberg in the turbulence of patients’ plasma proteome. Thus, the elucidation of plasma proteome of the H7N9 patients will shed light on more information regarding the development of this disease. In this study, we analyzed the plasma proteome at different postinfection stages of a family cluster, comprising a H7N9 patient and an asymptomatic case. Comparing the numbers of the differentially regulated proteins for the same patient as shown in Table 1, they were distinct across all three time points (the interval between two contiguous time point is 1 week), indicating that the protein changes that occur early

**Table 2: Proteins that show up- and down-ward tendency in the sequential plasma samples of both patients***

| Trend                | The patient                                                                 | The asymptomatic one                                           |
|----------------------|----------------------------------------------------------------------------|----------------------------------------------------------------|
| Upward tendency      | 1314244 (C4B), 4826762 (HP), 13540563 (CFHR5), 33319050 (Ig heavy chain variable region, VH3 family, partial), 194386718 (unnamed protein product), 197116005 (Ig heavy chain variable region, partial), 219566101 (Ig heavy chain, partial) | 5051025 (Ig gamma heavy chain variable region), 21669515 (Ig lambda light chain region), 221044808 (unnamed protein product) |
|                      | 177870 (A2M precursor), 284179 (Ig heavy chain V region), 4557871 (TF precursor), 5174411 (CD5L), 119608994 (PZP) | 177870 (A2M precursor), 284179 (Ig heavy chain V region), 4557871 (TF precursor), 5174411 (CD5L), 119608994 (PZP) |
| Downward tendency    | 29446 (unnamed protein product), 21669285 (Ig lambda light chain VLJ region, partial), 146424184 (APOC4) | 177870 (A2M precursor), 284179 (Ig heavy chain V region), 4557871 (TF precursor), 5174411 (CD5L), 119608994 (PZP) |
|                      | 5051025 (Ig gamma heavy chain variable region), 21669515 (Ig lambda light chain region), 221044808 (unnamed protein product) | 177870 (A2M precursor), 284179 (Ig heavy chain V region), 4557871 (TF precursor), 5174411 (CD5L), 119608994 (PZP) |

In total: 10

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*Data are GI number (protein name) of the proteins, unless otherwise indicated. Ig: Immunoglobulin, GI: GenInfo identifier

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**Figure 3:** An upward or downward expressing pattern in the differential protein profiles according to postinfection time points. (a and b) The results of the index case. (c and d) The results of the second case.
after getting infection are generally not sustained for longer than 1 week p.i. Cheung et al. have studied the proteome of primary human monocyte-derived macrophages infected with highly pathogenic H5N1 influenza virus and compared the temporal response at different time points after infection. They found that even at 1 h after viral infection, there was significant perturbation of the proteome induced by H5N1, and the protein changes were generally not sustained for longer than 6 h.\[8\] In our study, there were differences not only in the differential protein profiles of the three time points in each case but also between that of the two cases at the same postinfection time point. It provided huge information to better understand the body reaction after getting infection of H7N9.

Based on GO analysis, the biological processes, in which the differentially expressed proteins of each stage were enriched, partially reflected important features about the antivirus mechanism of the body. Most terms of biological processes were identical of the time points of the two cases except those of the 1-week time point of the second case. The enriched terms mainly focused on the response to stimulus, immune response, blood coagulation, lipid transport, and cell adhesion. It is similar with the previous study, which found that autoantibodies include anti-DNA antibody and anti-nuclear factor were most frequently detected in the influenza cases. Moreover, the levels of complement components increased immediately in the early stage after H7N9 virus infection. As shown in other study, levels of C4 and C9 also were elevated in most patients.\[9\] Blood coagulation was found in all time points of both index case and the second case. Similar results were also observed in the previous studies about H1N1 virus infection. H1N1 virus induced release of microparticles from platelets, surface receptor activation, as well as lipid mediator synthesis. Although the platelet activation triggers during H1N1 infection remains elusive, researchers inferred that immune complexes may contribute to H1N1-induced platelet activation, considering the complexes in H1N1 pandemic were reported playing a pathogenic role.\[10,11\] Therefore, the mechanism of blood coagulation emerged in the plasma of H7N9 patients may be analogous to that of H1N1 infection, which also needs further confirmation. Besides, lipid transport appeared in the plasma proteome of both cases and lasted for 3 weeks after H7N9 infection, indicating that the lipoproteins

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**Figure 4:** Subnetworks of the proteins with time-dependent expression pattern analyzed using STRING tool. (a and b) Network modules of the time-dependent proteins for the index case and the second case, respectively. (c and d) Network modules of the specific time-related proteins in the index case and the second case, respectively. Nodes and links refer to proteins and protein interactions, respectively.
may play an essential role in the antiviral process. For the 1-week time point of the second case, no significant terms were enriched by PANTHER, as there were some unknown proteins identified without any information in the database. These unknown proteins may play an important role in why this case did not develop any symptoms, and its elusive mechanism still needs a further demonstration.

For each case, there were a certain number of proteins with characteristic changing patterns within the sequential plasma samples. All of these representative proteins were of great value including proteins lacking functional annotation as exceptions. Some of the proteins were concurrent in both cases, with the same or opposite changing trend. Proteins including C4B, HP, and CFHR5 had an upward changing trend in the sequential plasma of the two cases whereas APOC4 had a downward trend. These proteins with same changing trend may be related with virus infection. For example, C4B (complement component 4B) is a trimer, providing a surface for interaction between the antigen-antibody complex and other complement components. It is the basic form of complement 4, part of the classical activation pathway. C4B levels were increased in the plasma samples of both Y and YM, in accordance with the previous study which also found that there was an increase in C4 levels during infection with influenza A virus.[12]

Proteins including A2M, TF, CD5 L, and PZP had a downward trend in the index case and had an upward trend in the second case. For instance, A2M (alpha-2-macroglobulin) is a protease inhibitor and cytokine transporter. It inhibits many proteases including trypsin, thrombin, and collagenase. Using a proteomic approach in conjunction with beads that bind alpha 2, 6-sialylated glycoprotein, researchers determined that A2M and an A2M-like protein are essential components in salivary innate immunity against hemagglutination mediated by a clinical isolate of swine origin influenza A virus.[13] Furthermore, during inflammation, reactive oxygen and nitrogen species can cause stress by directly or indirectly breaking covalent bonds in DNA, proteins, and lipids.[14] Therefore, it is widely recognized that imbalances in the lung antioxidant system can lead to oxidative stress, which in its most severe form, potentiates influenza-induced morbidity and even mortality.[15] As an antioxidant protein, transferrin (TF) that works like other antioxidant enzymes, can protect lung cells against reactive oxygen species stress.[16] Interestingly, in our proteomic data, either A2M or TF had an opposite trend in the sequential plasma of the index patient and the second case (both were downward in the index patient and upward in the second case), which may partially interpret why the index one developed pneumonia whereas the second case did not have any symptoms.

Furthermore, we performed the network analysis of these proteins with time-dependent expression pattern. Although the core proteins in the network of the index case and the second case were not entirely the same, most of them were members of the apolipoprotein family such as APOC1, APOC3, and APOC4. To the best of our knowledge, there was no other research on the plasma proteomic changes of H7N9 patients yet, but there were several researches on the differentially expressed proteins using infected human cells with H7N9 virus.[17,18] Ding et al. have analyzed the differentially expressed proteins in A549 cells infected with H7N9 virus at 24, 48, and 72 h pi and also performed network analysis of these proteins. The proteins in the central area of their network were CAPZA1, PCBP1, and EIF5A, and the proteins which were considered to be crucial in the process of H7N9 infection were OAT and PAFAH1B2. As the researchers focused on the differentially expressed proteins at each postinfection time point separately, their results were inconsistent with our findings which analyzed the proteins with time-related trend after H7N9 infection.[17] Furthermore, using the plasma samples from the H7N9 patient, our results might get more insight into the development of the disease.

Apolipoproteins were almost exclusively bound to lipoproteins, mainly very-low-density lipoproteins (VLDL) and high-density lipoprotein (HDL). In normotriglyceridemic plasmas, APOC1 is found predominantly in HDL, whereas APOC2 and APOC3 are about equally distributed between VLDL and HDL, APOC4 is found predominantly (about 80%) in VLDL, and APOC5 is a component of HDL.[19-21] Normal HDL can prevent the formation of the low-density lipoprotein (LDL)-derived oxidized phospholipids or inactive them after they are formed. In addition, it is proposed that LDL-derived oxidized phospholipids and HDL may be part of a system of nonspecific innate immunity.[22] Van Lenten et al. infected mice with influenza virus strain A/WSN/33 and found that the ability of HDL from infected mice to inhibit LDL oxidation and LDL-induced monocyte chemotactic activity in human artery wall cell cocultures decreased with time after inoculation.[23] APOCs were involved in the networks with respect to the H7N9 virus infection, implying that the HDL and VLDL probably played an important role in the progression of this disease.

There were several limitations that should be considered when interpreting findings of our study. First, we need amplify the complexity of the plasma proteome for mining influenza-related biomarkers in plasma. It is acknowledged that high-abundance proteins in the circulating plasma, such as albumin and apolipoprotein, hamper the identification of low-abundance proteins that are more clinically meaningful. Most notably, cytokines closely linked with response to H7N9 virus infection are clustered to the low abundance proteins.[24] Given that we excluded the high-abundance proteins at the beginning of the study and inevitably removed few low-abundance proteins associated with the high-abundant proteins targeted for depletion. Hence, the differential protein profiles generated by this strategy are not so perfect. Second, although our study provided a large body of potential biomarkers, it still lacks sufficient verifcation, as H7N9 patients found in Beijing were few. We did not observe all the cytokines that found in the plasma of H7N9 patients in other studies. In our study, the
index case was a 7-year-old girl with mild symptoms, whereas most of the patients in the study of Zhejiang Province were all adult patients with severe symptoms. The immune status of the patients was diverse due to the different physiological background and disease severity, thus leading to distinct plasma proteomes.

Conclusions

To conclude, an avian influenza A (H7N9) virus infection-related plasma database was generated with the nonredundant proteins identified in the current study. Importantly, there were some proteins characterized with time trend postinfection in the sequential plasma of both cases, providing huge information for mining candidate biomarkers related with H7N9 virus infection and clearance, and additional validation studies would further be launched.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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