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A new perspective on C-reactive protein in H7N9 infections

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SUMMARY

Objectives: The avian influenza H7N9 virus can cause cytokine overproduction and result in severe pneumonia and acute respiratory distress syndrome. Many studies have focused on hypercytokinemia during avian influenza infection. This study examined the association between C-reactive protein (CRP) and cytokines.

Methods: The plasma cytokine and chemokine profiles of 57 H7N9 patients were investigated using a multiplex immunoassay. The CRP levels of patients with H7N9 and patients with H1N1 were also compared. Further, the association between cytokines and CRP in H7N9 infections was explored.

Results: Compared with H1N1 virus, it was found that H7N9 virus induced higher expression of CRP, leading to cytokine storms. Several cytokines, including MIP-1β, MCP-1, IP-10, and IL-6, were observed to have significantly positive relationships with CRP levels, whereas IL-17A was negatively associated with CRP levels.

Conclusions: These findings suggest that CRP may be used as an early indicator to identify high-risk patients, to assess disease progression, and to determine the development of hypercytokinemia.

1. Introduction

Influenza A H7N9, a newly emerging avian virus that was first identified in China in March 2013, has resulted in 571 laboratory-confirmed cases of human infection and 212 reported deaths.[1] In addition to the common features of fever and cough, H7N9 virus-infected patients present with severe pneumonia and acute respiratory distress syndrome (ARDS), accompanied by fatal outcomes.[2,3] Similar to H5N1, H7N9 has been shown to cause hypercytokinemia in both plasma and lung tissues.[4,5] Hypercytokinemia is considered both an immune response and a critical pathogenic factor.[6,7] Previous studies have reported that several specific cytokines are highly associated with disease severity and outcomes.[8]

C-reactive protein (CRP) is synthesized primarily by the liver,[9] and is one of the non-specific acute-phase proteins produced in response to most forms of infection, inflammation, and tissue injury. Thus, this protein has been considered a non-specific biomarker for early diagnosis and prognostic measurements.[10,11] Specifically, CRP exhibits superior diagnostic value for bacterial infections with high plasma concentrations. However, CRP levels remain normal or increase only slightly during most viral infections.[12] As reported previously, patients with H7N9 have significantly higher CRP levels compared to patients with H1N1,[13] another subtype of avian influenza virus. Moreover, significantly higher CRP expression in patients with H7N9 positively correlates with disease severity.[14]

Previous studies have compared the roles of CRP, angiotensin II,[15] and several cytokines[8] in predicting the progression and outcome of influenza infections and have suggested that angiotensin II is a good biomarker and that specific cytokines are good predictors of the outcome. There is no doubt that the early identification of high-risk cases and the monitoring of illness progression greatly contribute to appropriate clinical decision-making. However, current cytokine and angiotensin II measurement techniques are either costly or complicated, thus easier examination methods are required to identify high-risk cases. Moreover it was considered of interest to determine the mechanism by which the virulent influenza virus H7N9 causes a

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high level of plasma CRP and whether a possible relationship exists between CRP and these cytokines. This study investigated inflammatory cytokine and CRP levels in patients with H7N9 and H1N1 infections and analyzed the possible correlation between cytokines and CRP.

2. Patients and methods

2.1. Patients and associated procedures

From April 2013 to February 2014, 82 patients with laboratory-confirmed H7N9 infections were recruited at the First Affiliated Hospital, College of Medicine, Zhejiang University. Fourteen patients with H1N1 virus in Beijing from December 2012 to February 2013 were also recruited, as well as six healthy volunteers as controls. The presence of H7N9 and H1N1 viruses was confirmed using protocols described previously.[14,16,17] Plasma samples were collected within 2 days of admission. Patient sputum samples or pharyngeal swabs were collected on the same day. Twenty-five patients with H7N9 were excluded because their plasma samples were collected more than 10 days after the onset of fever. Data on clinical characteristics were collected on the same day as plasma sampling (Table 1). Blood samples were obtained using ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes and centrifuged to collect plasma. Viral load was detected by TaqMan real-time reverse transcription PCR targeting the influenza A H7N9 virus subtype-specific H7 gene or influenza A H1N1 virus subtype-specific H1 and N1 genes using standard thermal cycling conditions.[18,17,19] All of the specimens were stored at −80 °C until analysis. This study complied with the necessary ethical guidelines and was approved by the Research Ethics Board of the First Affiliated Hospital of Zhejiang University. All participants or their guardians signed an informed consent form before enrolment.

2.2. Measurement of cytokine and chemokine concentrations in patient plasma

A 27-plex assay kit was used to measure the concentrations of plasma cytokines (Bio-Plex Pro Human 27-plex cytokine group I; Bio-Rad, CA, USA); the manufacturer's protocol was followed. The 27-plex assay contains the following cytokines: basic fibroblast growth factor (FGF), EOTAXIN, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), interleukin (IL)-10, IL-12 (p70), IL-13, IL-15, IL-17A, IL-1 receptor antagonist (Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IFN-γ-induced protein 10 (IP-10), IL-1β, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β, platelet-derived growth factor BB (PDGF-BB), RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF). Samples were analyzed using a MAGPIX system (Luminex Corporation, Merck Millipore, Temecula, CA, USA), and the data were processed using Bio-Plex Manager 6.1 software (Bio-Rad). Cytokine/chemokine values that were detected outside of the standard range were flagged as high or low out-of-range (OOR), and the highest or lowest value on the standard curve was used instead of the OOR measurement. IL-15 and IL-1β measurements were excluded because more than 50% of the values were OOR below the standard curve.

Table 1
Characteristics of the patients with H7N9 and H1N1 infections included in the study

Characteristics	H7N9	H1N1
Number	57	14
Age, years, median (range)	62 (21–86)	54 (26–85)
Male sex, *n*/total *n* (%)	36/57 (63.2%)	9/14 (64.3%)
History of confirmed poultry contact, *n*/total *n* (%)	28/57 (49.1%)	NA
History of confirmed contact with a patient with H7N9 infection, *n*/total *n* (%)	2 /57 (3.5%)	NA
Antiviral therapy before sampling, *n*/total *n* (%)	41/57 (71.9%)	9/14 (64.3%)
ARDS (%)	37/57 (64.9%)	3/14 (21.4%)
Days from onset of fever to sampling	6.7 ± 2.2	7.75 ± 3.38
CRP, mg/l	84.0 ± 65.2	11.37 ± 6.70
White blood cell count, $\times 10^9$/l	5.6 ± 4.4	5.76 ± 2.82
Neutrophils, $\times 10^9$/l	4.8 ± 4.1	4.17 ± 2.80
Median Ct value of initial viral load (*n*)	30 (33)	30 (12)
Clinical outcome of death, *n*/total *n* (%)	18/57 (31.6%)	0 (0)

ARDS, acute respiratory distress syndrome; CRP, C-reactive protein; Ct, cycle threshold; NA, not available.

2.3. Measurement of CRP in patient plasma

CRP was detected using a Beckman-Coulter IMMAGE 800 Immunohistochemistry System and Beckman-Coulter reagents (Beckman-Coulter, Brea, CA, USA).[20]

2.4. Statistical analysis

Results are expressed as the mean ± standard deviation, or the median, as appropriate. Differences between groups were evaluated by unpaired *t*-test or one-way analysis of variance (ANOVA). Pearson's correlation coefficient and Spearman's rank correlation coefficient were used for linear correlation analysis. Statistical analyses were performed using IBM SPSS Statistics version 19.0 software (IBM Corp., Armonk, NY, USA), and figures were generated with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the study subjects

The characteristics of the patients are shown in Table 1. The median age of the patients with H7N9 infection was 62 years, and 36 patients (63.2%) were male. Twenty-eight patients (49.1%) had a contact history with poultry, and two patients had a contact history with a patient infected with H7N9. The mean interval from fever onset to sampling was 6.7 ± 2.2 days for the patients with H7N9 and 7.75 ± 3.38 days for the patients with H1N1. The patients with H7N9 were more likely to progress to ARDS (64.9%) than the patients with H1N1 (21.4%). Forty-one (71.9%) patients with H7N9 were treated with antiviral drugs before sampling compared to 64.3% of patients with H1N1. The CRP levels were much higher in patients with H7N9 infection than in patients with H1N1 infection (84.0 ± 65.2 compared to 11.37 ± 6.70 mg/l, $p < 0.01$). However, the white blood cell and neutrophil counts were within the normal range in both groups (Table 1). The median cycle threshold (Ct) values of the H7N9 and H1N1 viruses were both 30. The overall mortality of patients with H7N9 infection was 31.6%, and that for patients with H1N1 was 0%.

3.2. Comparison of plasma CRP levels between H1N1 and H7N9

Based on clinical outcomes, the patients with H7N9 were divided into two groups, a survivor group and a non-survivor group. The CRP plasma levels of these two groups were compared with that of the H1N1 group. The CRP concentration of the H1N1 group was significantly lower than those in the survivor ($p < 0.01$)

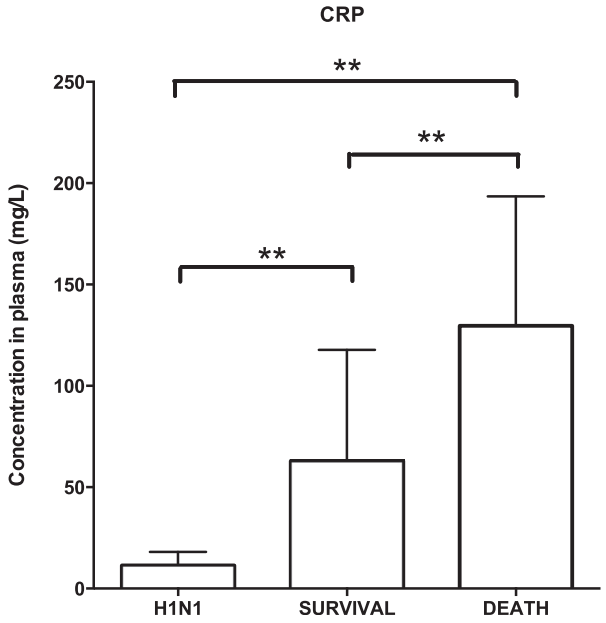

Figure 1. Comparison of plasma CRP levels between patients with H1N1 and patients with H7N9. H1N1, H1N1-infected group; SURVIVAL, H7N9-infected survivors; DEATH, H7N9-infected non-survivors. The data represent the mean ± standard deviation (H1N1, $n = 14$; SURVIVAL, $n = 39$; DEATH, $n = 18$; $^{**}p < 0.01$).

and non-survivor H7N9 groups ($p < 0.01$) (Figure 1). Patients with H7N9 who died had much higher concentrations of CRP (129.6 ± 63.9 mg/l) than the patients who survived (63.0 ± 54.8 mg/l) ($p < 0.01$).

3.3. Correlation of CRP with cytokines in patients with H7N9 infection

A linear correlation analysis of CRP with the expression of all measured cytokines was performed for the H7N9 infection group (Table 2). The concentrations of seven cytokines/chemokines were significantly correlated with CRP levels. In addition, the CRP levels in patients with H7N9 infection within 10 days of fever onset demonstrated a strong positive linear correlation with MIP-1β ($r = 0.5507$, $p < 0.0001$) (Figure 2B), modest positive linear correlations with MCP-1 ($r = 0.3885$, $p = 0.0028$), IP-10 ($r = 0.3044$, $p = 0.0213$), and IL-6 ($r = 0.3041$, $p = 0.0215$) (Figure 2C–E), and modest negative linear correlations with IL-17A ($r = -0.3020$, $p = 0.0224$), RANTES ($r = -0.3385$, $p = 0.0100$), and EOTAXIN ($r = -0.2979$, $p = 0.0244$) (Figure 2F–H). The quartiles and minimum and maximum values of the seven cytokines listed above in the H7N9 infection, H1N1 infection, and healthy groups are shown in Figure 2A. MCP-1, IP-10, IL-6, and IL-17A levels in the H7N9 infection group were significantly elevated compared to those in the H1N1 virus-infected and healthy groups. The plasma levels of MIP-1β and RANTES were also higher in the two patient groups than in the healthy individuals. Furthermore, the concentration of plasma MIP-1β was higher in H7N9-infected patients than in H1N1-infected patients, although this trend did not pass hypothesis testing. Furthermore, EOTAXIN levels did not differ among the three groups. Correlations between CRP and the other measured cytokines in H7N9 infection were not significant ($p > 0.05$) (Table 2).

3.4. Correlation of seven cytokines with Ct values of viral load in patients with H7N9 infection

Based on the above findings, linear correlation analysis of these seven cytokines with Ct values of H7N9 virus was performed (**Supplementary Material**, Table S1). Plasma levels of MIP-1β, MCP-1, IP-10, and IL-6 were highly associated with Ct values of the H7N9 virus in a small H7N9-infected patient group ($r = -0.411$, $p = 0.017$; $r = -0.481$, $p = 0.005$; $r = -0.557$, $p = 0.001$; $r = -0.394$, $p = 0.023$, respectively; $n = 33$). However, there were no significant correlations between the other three cytokines and Ct values.

Table 2
Correlation of CRP with cytokines in patients with H7N9 infection

Cytokine	Pearson/Spearman *r*	*p*-Value
Basic FGF[b]	−0.1915	0.1536
EOTAXIN[b]	−0.2979	0.0244[c]
G-CSF[b]	0.02314	0.8643
GM-CSF[b]	−0.08345	0.5371
IFN-γ[b]	−0.1378	0.3067
IL-10[b]	−0.08414	0.5338
IL-12 (p70)[b]	−0.2326	0.0817
IL-13[b]	−0.1340	0.3203
IL-17A[a]	−0.3020	0.0224[c]
IL-1Ra[b]	−0.06116	0.6513
IL-2[b]	−0.2601	0.0507
IL-4[b]	−0.2018	0.1322
IL-5[b]	−0.2254	0.0918
IL-6[b]	0.3041	0.0215[c]
IL-7[b]	−0.2210	0.0986
IL-8[b]	0.1008	0.4558
IL-9[b]	−0.1889	0.1593
IP-10[b]	0.3044	0.0213[c]
MCP-1[a]	0.3885	0.0028[c]
MIP-1α[b]	0.07860	0.5611
MIP-1β[a]	0.5507	<0.0001[c]
PDGF-BB[b]	−0.2482	0.0627
RANTES[b]	−0.3385	0.0100[c]
TNF-α[b]	−0.2039	0.1281
VEGF[b]	−0.1112	0.4101

CRP, C-reactive protein; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
[a] Calculation of the Pearson correlation coefficient of CRP with the cytokine (in $\log_{10}$).
[b] Calculation of the Spearman rank correlation coefficient of CRP with the cytokine.
[c] $p < 0.05$.

4. Discussion

This study investigated both inflammatory cytokine and CRP levels during H7N9 infections and explored their possible correlation. By comparing CRP levels in different groups, it was noted that patients infected with H7N9 were more likely to exhibit high CRP expression levels; however, patients with high levels of CRP had poor outcomes. It was also observed that the levels of several cytokines, including MIP-1β, MCP-1, IP-10, IL-6, IL-17A, RANTES, and EOTAXIN, were significantly associated with CRP levels.

Hypercytokinemia (also known as a 'cytokine storm') is characterized by the over-production of various proinflammatory cytokines and plays an important role in disease severity and fatal outcomes in patients with H5N1 infection.[6] Compared with H1N1 infection, H7N9 virus infection tends to induce higher cytokine expression, resembling the cytokine storm observed in H5N1 infection.[18] Similarly, hypercytokinemia also correlates positively with the deterioration of H7N9 infection, indicating that advanced detection of a cytokine storm and the determination of severe cases are necessary. In this work, it was hypothesized that CRP would be a simple and convenient but non-functional marker for the early prediction of potential cytokine storms.

MCP-1 (CCL-2) and MIP-1β (CCL-4) belong to the CCL family and are two chemokines that primarily affect the recruitment of monocyte/macrophages to inflammatory sites,[21] thus mediating

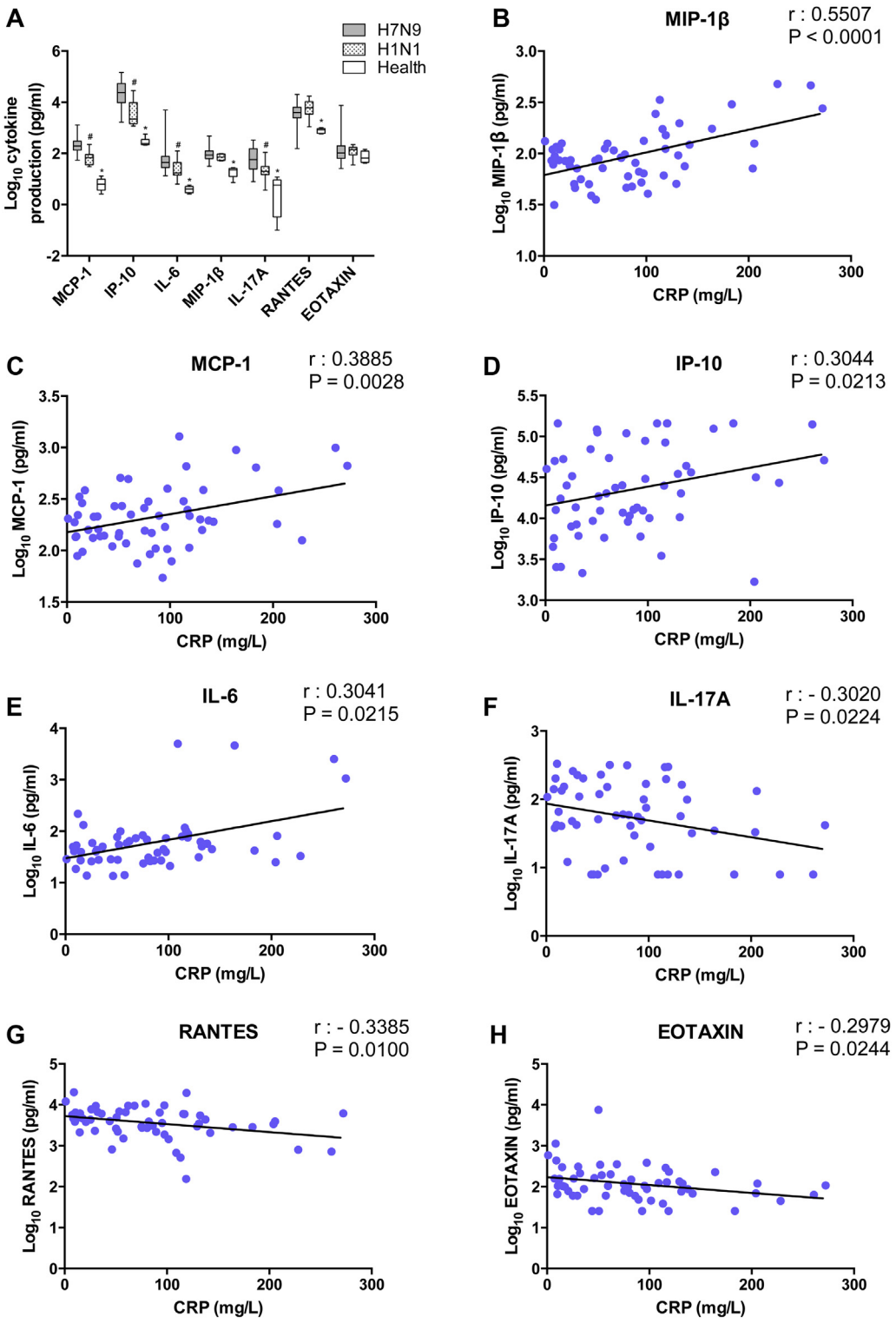

Figure 2. (A) Plasma MIP-1β, MCP-1, IP-10, IL-6, IL-17A, RANTES, and EOTAXIN levels in the H7N9 infection, H1N1 infection, and healthy groups within 10 days of fever onset. Quartiles and minimum and maximums values are presented. *Comparison between the healthy group and the H7N9 group. #Comparison between the H7N9 and H1N1 groups, $p < 0.05$. (B)–(H) Correlation between CRP and plasma cytokine levels (MIP-1β, MCP-1, IP-10, IL-6, IL-17A, RANTES, EOTAXIN) in patients with H7N9 infection and fever. The Pearson correlation coefficient (r), Spearman rank correlation coefficient (r), and p-values are provided in each graph.

host innate immunity and expanding inflammatory effects. MCP-1 and MIP-1β levels increase in patients with H7N9.[14,18] In this study, it was found that MCP and MIP-1β had significant positive relationships with CRP, and the latter displayed a much stronger relationship. Furthermore, both of these were highly related to the initial viral load in H7N9 infection (**Supplementary Material**, Table S1), which has been reported previously for H5N1 and H7N9 infection.[6,14] MIP-1β levels are higher in patients with H7N9 than in patients with H5N1.[18] Moreover, MIP-1β levels correlate positively with poor clinical outcomes.[4] Because CRP levels are also related to disease severity, the correlation between chemokines and CRP may be obvious. However, CRP can also mediate the

secretion of CC chemokines from monocytes, including MCP-1 and MIP-1β,[22] leading us to hypothesize that CRP, the primary acute phase protein, might participate in the pathogenesis of H7N9 infections as a secondary inducer of chemokines in addition to direct stimulation by the influenza virus.

IP-10 is a proinflammatory cytokine that is secreted by a variety of cells, including monocytes, fibroblasts, endothelial cells, and hepatocytes.[23] As a member of the CXC chemokine family, IP-10 can recruit and activate T-cells, monocytes/macrophages, natural killer (NK) cells, eosinophils, and dendritic cells.[23–26] IP-10 is elevated in patients with H5N1 infection, particularly in patients who die,[6] and a similar elevation has also been detected in patients with H7N9 infection, with severe cases exhibiting significantly higher levels of IP-10.[7,8] Moreover, the IP-10 plasma levels correlate with the influenza virus load in patients with both H5N1 and H7N9 infections,[6,8] which was also found in the present study (**Supplementary Material**, Table S1). Patients with H7N9 infection share unusually high CRP plasma levels with H5N1 patients, a phenomenon not observed in patients with H1N1 infection.[13] Therefore, the relationship between IP-10 and CRP was studied and a positive correlation was observed. As reported previously, IP-10 plays a critical role in the earliest stages of acute lung injury.[27,28] In this study, simultaneously elevated CRP and IP-10 levels were detected, suggesting that IP-10 may cause the increased expression of plasma CRP.

IL-6 levels are increased in patients with H7N9 infection and are significantly higher in patients with severe infections. High levels of IL-6 have also been associated with poor clinical outcomes.[4] In this study, a positive correlation between CRP and IL-6 levels was observed. IL-6 induces CRP gene expression in hepatocytes.[29] Moreover, IL-6-blocking therapies can restore normal plasma CRP levels in chronic inflammatory diseases,[30] and the underlying mechanism has been reported.[31] It was considered that after infection with H7N9, increased IL-6 levels may also induce CRP expression.

IL-17A (usually referred to as IL-17) was initially described as a characteristic cytokine secreted by Th17 lymphocytes, NK T-cells (NKT), γδ T-cells, and CD8+ T-cells.[32,33] IL-17A stimulates proinflammatory chemokines and recruits neutrophils into the airway.[34] IL-17A plays a significantly defensive role in various infections,[33] and is involved in asthma.[35] In patients with chronic obstructive pulmonary disease (COPD), IL-17A levels increase as the disease progresses, and the serum levels positively correlate with CRP.[36] In patients with H7N9 infection, high plasma concentrations of IL-17A were detected, consistent with previous data.[7] However, intriguingly, IL-17A was negatively correlated with CRP in the present study. Previous studies have indicated that IL-17A primarily promotes neutrophilic inflammation.[37–39] Additional studies are needed to clarify the exact role of IL-17A in H7N9 infection and the underlying mechanism between IL-17A and CRP levels.

RANTES (CCL5) is a small 68-amino acid protein that belongs to a rapidly growing chemokine family. It can be strongly induced by viral and bacterial infections and recruits T-cells, dendritic cells, eosinophils, NK cells, mast cells, and basophils to sites of inflammation and infection.[40] EOTAXIN is another chemokine with potent and selective agonist activity for CC chemokine receptor 3, which can attract eosinophils to sites of inflammation. In the present study, it was observed that the CRP levels 10 days from fever onset in patients with H7N9 infection had a negative linear correlation with both RANTES and EOTAXIN, which was contradictory to the hypothesis. For this reason, the two chemokines were compared between the groups. There was no significant difference in the plasma levels of RANTES or EOTAXIN between the H7N9- and the H1N1-infected patients (Figure 2A), which is consistent with previous findings in H7N9- and H5N1-infected patients.[6,7] It is supposed that the negative correlations of CRP with RANTES and EOTAXIN may result from significant differences without biological relevance, which should be confirmed further in a larger number of subjects.

In accordance with previous studies,[13,14] H7N9 patients presented with high levels of CRP. This elevated acute phase protein was related to disease severity, indicating an underlying secondary bacterial infection. Although cytokine storms reflect a host defense response against pathogens, the overwhelming levels of activated factors could cause immunity injury and contribute to pathogenesis. Nonetheless, the immunopathological mechanism of H7N9 infection remains unclear. It was hypothesized that in addition to various cytokines, CRP also plays an important role in the progression of this disease. Certain connections between CRP and cytokines exist that form a complicated inflammatory network, suggesting that this acute-phase protein might reflect the release of inflammatory factors to a certain extent. Moreover, no correlation was found between CRP and the initial viral load in H7N9 infection (data not shown), which indicates that the plasma level of CRP was not influenced by the initial viral load. While the condition of patients with H7N9 is complicated and variable, CRP, as a conventional clinical indicator, could be a simple and convenient marker for the early prediction of potential cytokine storms, particularly when cytokine detection is unavailable. This marker will contribute to the early identification of high-risk cases, further assessment of cytokine profiles of severe cases, and the development of an appropriate therapeutic plan, such as anti-cytokine treatment.

There are a few limitations in relation to this study. Samples were collected at only one time-point. Continuous monitoring and comparison of CRP and cytokine levels was also lacking. The standard deviation of CRP was large because of the small sample size and variable disease conditions. Further studies are necessary to determine the exact role of hypercytokinemia in H7N9 and its association with CRP.

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Conflict of interest: The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2016.01.009.

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