Inflammatory molecules expression pattern for identifying pathogen species in febrile patient serum

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Abstract. Inflammatory molecules, such as cytokines and chemokines, have been considered markers for bacterial or viral infection in serum of patients in numerous studies. The aim of the present study was to investigate whether we were able to identify the pathogen species through patterns of inflammatory molecules. A total of 132 patients with elevated body temperature (tympanic temperature, >38.3˚C) were recruited for this study. The concentrations of various inflammatory molecules in the patients' serum were evaluated using a cytometric bead array. Higher concentrations of interleukin (IL)-6 and IL-8 were detected in bacterial infection groups (patients with positive and negative blood cultures), as compared with the viral infection group. Viral infection (including influenza and dengue viral infections) was associated with higher concentrations of interferon-γ-inducible protein 10 (IP-10), as compared with the bacterial infection group. In addition, IL-8 levels in the gram-negative bacteria group were higher, as compared with the gram-positive bacteria group. However, IL-8 was insufficient for bacterial species identification. By contrast, dengue virus infection induced the highest serum level of IP-10 among all groups. In conclusion, detection of the patterns of inflammatory molecules may aid the subsequent management and treatment modalities in hospitals, although evaluation of these molecules alone may be insufficient for identifying the pathogen species.

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Introduction

Fever is among the most common presenting symptoms in emergency department hospital cases; however, fever may be a symptom of numerous diseases (1). Pathogen infection is one of the most common causes of induced fever. In addition to the basic history assessment and physical examination of patients, emergency physicians typically require more information, such as laboratory test results and imaging examination, to diagnose the causes of fever-associated diseases (2).

In clinical settings, certain laboratory tests have been used to facilitate the diagnosis of severe bacterial infection, such as white blood cell (WBC) count, C-reactive protein (CRP) and procalcitonin (3,4). As a host immune system recognizes pathogens and induces production of inflammatory molecules to resist pathogens, numerous cytokines and chemokines are used as biomarkers in laboratory research for the diagnosis and prognostic prediction of infectious diseases (5-13). For example, studies have shown that inflammatory cytokines levels are altered as a result of a variety of infections, including interleukin (IL)-1, IL-6, IL-10, IL-13, IL-18 and tumor necrosis factor-alpha (TNF-α) (5-8). Elevating plasma concentration of cytokines such as IL-6 and IL-10 may serve as a diagnostic marker of sepsis and is correlated with severity and survival of sepsis patients (9,10). In addition, certain chemokines, such as Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), interferon-gamma-inducible protein 10 (IP-10) and CXCL8/IL-8, are associated with sepsis (11-13). These markers may provide important information for the early diagnosis of diseases.

Immune system recognizes different pathogens with particular characteristics, such as lipopolysaccharides on gram-negative bacteria, peptidoglycan on gram-positive bacteria and single-stranded and double-stranded RNA in RNA viruses, using pattern-recognition receptors (14). Each characteristic triggers distinct downstream pathways and the production of inflammatory molecules (15). Since different pathogens induce different immune responses, we aimed to determine whether the pathogen species could be identified by patterns of serum inflammatory molecules. In the present study, we examined whether bacterial and viral infections, gram type of bacteria, different species of bacteria, or different

types of virus could be characterized by measuring a variety of cytokines and chemokines using a cytometric bead array. The expression pattern of seven cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α and IFN-γ) and five kinds of chemokines [IL-8, RANTES, monokine induced by interferon-γ (MIG), monocyte chemoattractant protein 1 (MCP-1) and IP-10] were detected in fever patients.

Materials and methods

Patient enrollment. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital. Patients visiting the Emergency Department of Kaohsiung Medical University Hospital (Taiwan, China) with fever or high body temperature (tympanic temperature, >38.3˚C) were sequentially enrolled between May 2013 and October 2014. Patients received treatments and examinations according to emergency physicians' evaluation. If patients agreed and provided informed consent, an extra 10 ml blood would be collected to undergo cytokine test. Serum was separated by centrifugation at 1,600 x g for 20 min at 4˚C, divided in aliquots and immediately frozen (-80˚C) until the time of the assay. Clinical information including the patients' age, gender, culture result, laboratory data at the Emergency Department and diagnosis were collected by a research nurse.

Cytometric bead array. Cytokines and chemokines from the sepsis patients were analyzed using the Cytometric Bead Array for human Th1/Th2/Th17 Cytokine kit (cat no. 560484; including IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α and IFN-γ) and a Human Chemokine kit (cat no. 552990; including IL-8, RANTES, MIG, MCP-1, and IP-10; BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Data was acquired using a BD Accuri C6 flow cytometer (BD Biosciences) and the data were analyzed using FCAP Array™ version 3.0.1 Software (BD Biosciences) according to a standard concentration curve.

Statistical analysis. Differences between two independent groups were analyzed by Mann-Whitney U test. Comparisons between three groups were via use of the Kruskal-Wallis test with Dunn's multiple comparison test. All calculations were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Laboratory data. Enrolled febrile patients were divided into two groups: Clinically suspected viral infection and bacterial infection, according to initial diagnosis. C-reactive protein (CRP) level and white blood cell (WBC) count are crucial indicators of sepsis (1,2). High levels of CRP (12.81±17.78 vs. 124.91±90.44 mg/l; $P<0.0001$) and WBC counts ($4.93\pm2.5\times10^3$ vs. $7.84\pm5.28\times10^3$ cells/μl; $P<0.0001$) were detected in patients who were diagnosed as having bacterial infection compared with patients who were diagnosed with viral infection (Table I). Compared to viral infection, significantly higher levels of blood urea nitrogen (BUN), blood glucose, creatinine (Cr), platelet and significantly lower levels of hemoglobin (Hb) were observed in bacterial infection patients.

Association between viral and bacterial infection and pattern of inflammatory molecule expression. We further examined the inflammatory molecules in patients serum (Table II). Patients with suspected bacterial infection exhibited significantly higher levels of IL-6 (63.23±265 vs. 2,533±6,559 pg/ml, $P<0.0001$), IL-10 (14.27±16.75 vs. 205.11±741.85 pg/ml, $P=0.0101$), IL-17A (18.66±54.93 vs. 34.58±63.99 pg/ml, $P=0.0162$), TNF-α (0.52±1.37 vs. 19±100.22 pg/ml, $P=0.0007$), IL-8 (30.31±38.01 vs. 1,249±6,944 pg/ml, $P<0.0001$) and MIG (660.71±661 vs. 4,533±14,580 pg/ml, $P=0.0367$). The present results were comparable to those of previous reports (5-8).

Currently, bacteria identification is primarily dependent on culture diagnosis in hospitals. However, studies have shown that culture-negative patients account for 28-48% of severe sepsis patients in North American, French and Canadian intensive care units (16-18). There were ~40% of culture-negative cases in a pan-European study and 49% of culture-negative cases in sepsis patients in the United States (19,20). These studies suggest that methods development for pathogen species identification is a critical issue. In the present study, there were 47 culture-positive patients and 37 culture-negative patients. We investigated whether different patterns of inflammatory molecules could be observed between viral infection group, culture-negative group and culture-positive group, particularly between viral infection and culture-negative group (Fig. 1). There was a statistically significant difference in IL-6, IL-8 and IP-10 levels between the viral infection and culture-negative groups or viral infection and culture-positive groups (Fig. 1). Similar results were observed in serum CRP level and WBC count (Fig. 2). Notably, the IL-2 and MCP-1 levels in the culture-positive group were higher than those in culture-negative group. However, neither molecule could not distinguish viral infection from bacterial infection (Table II). The results suggest that IL-6, IL-8 and IP-10 may be suitable for distinguishing viral infection from bacterial infection, even though bacterial culture is negative.

Association between species of bacteria and pattern of inflammatory molecule expression. Although serum CRP level and WBC counts were markers of bacterial infection, they showed no significant difference between gram-negative and gram-positive groups in the culture-positive group in the present study (data not shown). IL-8 level in gram-negative bacteria infection was higher compared with gram-positive bacteria infection group among the 47 culture-positive patients though IL-6 and IP-10 level did not show statistical difference (Table III). According to the result of bacterial culture, serum from *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* infected patients were selected. However, the results might indicate that these molecules were not sufficient to identify the species of bacteria (Table IV).

Association between virus type and pattern of inflammatory molecule expression. In the viral infection group, influenza virus or dengue virus-infected patients were enrolled after initial diagnosis. IL-6 level in influenza virus infection group is higher compared with the dengue virus infection group. However, the IL-6 level in influenza virus group was

Table I. Patient characteristics and laboratory data.

Characteristic	Viral infection (n=54)	Bacterial infection (n=78)	P-value
Male/female	31/23	41/37	-
Age (years)	43.74±15.79	63.81±16.4	<0.0001
CRP (mg/l)	12.81±17.78 (n=49)	124.91±90.44	<0.0001
WBC ($10^3/\mu$l)	4.93±2.50	11.84±5.28	<0.0001
Platelet ($10^3/\mu$l)	149.74±58.3	203.97±99.43	0.0011
Hb	14.02±4.13	12.11±2.13	<0.0001
BUN	10.95±4.99 (n=35)	19.73±13.15 (n=73)	<0.0001
Glu	128.27±33.36	176.07±135	0.0496
Cr (mmol/l)	0.93±0.22 (n=44)	1.30±0.76 (n=76)	0.0190

Some data was undetectable in some patients. Data presented as the mean ± standard deviation. CRP, C-reactive protein; WBC, white blood cell; Hb, hemoglobin; BUN, blood urea nitrogen; Glu, glucose; Cr, creatinine.

Table II. Cytokines and chemokines level in viral infection and bacterial infection patients.

Expression (pg/ml)	Viral infection (n=54)	Bacterial infection (n=78)	P-value
IL-2	1.46±1.04	3.2±6.67	0.7680
IL-4	0.99±0.94	1.47±3.75	0.3510
IL-6	63.23±265	2,533±6,559	<0.0001
IL-10	14.27±16.75	205.11±741.85	0.0101
IL-17A	18.66±54.93	34.58±63.99	0.0162
TNF-α	0.52±1.37	19±100.22	0.0007
IFN-γ	9.54±19.16	16.80±39.61	0.8494
IP-10	1,944±1,299	666.72±766	<0.0001
MCP-1	493.31±679	4,533±14,580	0.3906
MIG	660.71±661	1,377±1,906	0.0367
RANTES	3,424±3,439	4,897±5,006	0.5866
IL-8	30.31±38.01	1,249±6,944	<0.0001

Data presented as the mean ± standard deviation. IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IP-10, interferon-γ-inducible protein 10; MCP-1, monocyte chemoattractant protein 1; MIG, monocyte induced by gamma interferon; RANTES, Regulated on Activation, Normal T cell Expressed and Secreted.

significantly lower (P<0.05) compared with the bacterial infection group (Table II). By contrast, serum from dengue virus infected patients contained significantly higher levels of IP-10 compared with serum from influenza virus infected patients (Table V). These results suggested that IP-10 levels may be used to distinguish influenza virus infection from dengue virus infection.

Discussion

Clinical laboratory data, including WBC count and CRP level, is rapid, sensitive and specific method for detecting bacterial infection (3,4). Platelet, BUN and Cr levels exhibited significant differences between suspected viral infection and bacterial infection patients. As patients with dengue fever, which affected platelet level, were recruited (21), platelet level was lower in viral infection patients than in bacterial infection patients. Anemia, hyperglycemia and elevated BUN and creatinine have been shown to be associated with bacterial infection (22,23); however, this may be due to patients selected in the clinically suspected bacterial infection group being older than patients in a viral infection group. IL-6, IP-10 and IL-8 may be potential markers for distinguishing viral infection from bacterial infection, although bacterial culture was negative. Furthermore, IP-10 level was highly correlated with dengue virus infection.

Culture-negative state is a commonly observed in patients diagnosed with sepsis (11-15). In the present study, serum CRP levels and WBC counts were sensitive and reliable indexes for distinguishing bacterial infection from viral infection. Numerous serum molecules, including IL-6, IL-8, IL-10 and TNF-α, have been reported to be markers of sepsis (5-7,9-11,24,25). Similar to the results of serum CRP level and WBC counts, significantly elevated concentrations of IL-6 and IL-8 were observed in fever patients in the bacterial infection group, regardless of culture-positive or

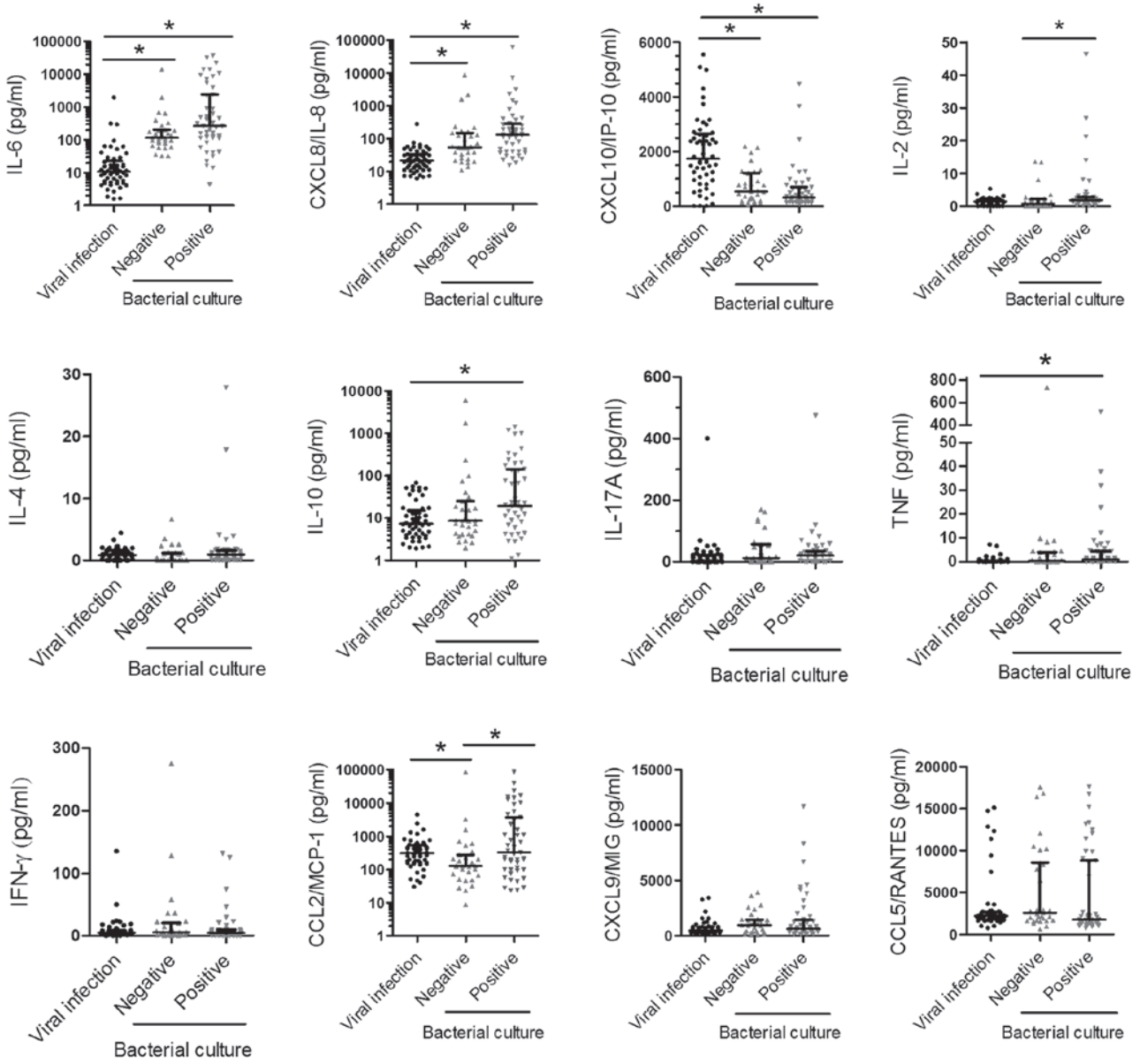

Figure 1. Inflammatory molecules in the serum of pathogen-infected patients. Comparisons among viral infection and bacterial infection (including culture-negative and culture-positive groups) were by use of the Kruskal-Wallis test with Dunn's multiple comparison test (*P<0.05). IL, interleukin; IP-10, interferon-γ-inducible protein 10; TNF, tumor necrosis factor; IFN-γ, interferon-γ; MCP-1, monocyte chemoattractant protein 1; MIG, monocyte induced by gamma interferon; RANTES, Regulated on Activation, Normal T cell Expressed and Secreted.

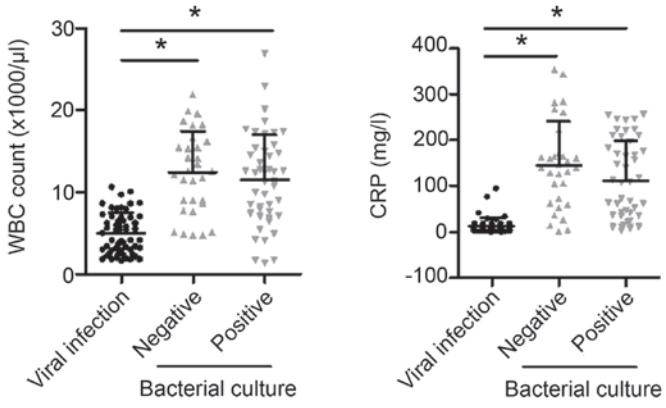

Figure 2. Comparison of CRP level and WBC counts between viral infection and bacterial infection groups. Data was analyzed by the Kruskal-Wallis test with Dunn's multiple comparison test (*P<0.05). WBC, white blood cell; CRP, C-reactive protein.

culture-negative status. IL-10 and TNF-α were observed to have significant differences between viral and bacterial infection modalities. However, differences in IL-10 and TNF-α were observed between culture-positive and viral infection groups, but not between culture-negative and viral infection groups. The results suggested that IL-6 and IL-8 were sensitive and specific markers of bacterial infection.

Accurate and rapid identification of bacterial species improve clinical outcome (26). However, blood culture typically requires 1-3 days to become positive for detection of bacteria. Previous reports suggest that microarray or quantitative polymerase chain reaction analyses may be a faster and sensitive method for clinical identification of bacterial infection (27). It is reported that procalcitonin, but not CRP and WBC counts, is associated with gram-negative bacteria (28). Due to the different components of the cell

Table III. Cytokines and chemokines level in gram-positive bacteria and gram-negative bacteria.

	Culture-positives (n=47)		
Expression (pg/ml)	Gram-negative (n=32)	Gram-positive (n=15)	P-value
IL-6	4,058±7,852	3,124±8,194	0.8284
IP-10	551.1±671.72	770.9±1,130	0.6896
IL-8	2,487±10,603	217±388	0.0293

Data presented as the mean ± standard deviation. IL, interleukin; IP, interferon-γ-inducible protein 10.

Table IV. Cytokines and chemokines level in different bacterial species.

Expression (pg/ml)	*Escherichia coli* (n=16)	*Klebsiella pneumoniae* (n=7)	*Staphylococcus aureus* (n=7)	P-value
IL-6	2,552±4,004	4,198±5,464	414±256.67	0.6630
IP-10	632.43±829	506.23±459.63	660.91±760	0.9822
IL-8	374.85±750	638.15±847	72.44±57.87	0.0688

Data presented as the mean ± standard deviation. IL, interleukin; IP, interferon-γ-inducible protein 10.

Table V. Cytokines and chemokines level in influenza virus and dengue virus.

Expression (pg/ml)	Influenza (n=18)	Dengue (n=36)	P-value
IL-6	167.3±441.4	11.20±15.56	<0.0001
IP-10	973.4±745.4	2,430±1,244	<0.0001
IL-8	40.78±60.72	24.76±15.51	0.6797

Data presented as the mean ± standard deviation. IL, interleukin; IP, interferon-γ-inducible protein 10.

walls of gram-positive and gram-negative bacteria, different patterns of immune responses may be induced *in vitro* (29,30). Therefore, we hypothesized that inflammatory molecules in the serum may provide information for identifying bacterial species. Gram-negative bacteria induce higher levels of IL-6, IL-10 and IL-8 compared with gram-positive bacteria in human monocytes (29-31). To the best of our knowledge, there are few studies reporting whether cytokines or chemokines could be markers to distinguish infection of gram-positive or gram-negative bacteria in patient serum. The present results revealed that IL-8 was the only molecule which exhibited a significant difference in expression between gram-negative and gram-positive bacteria. In the present study, bacterial species could not be identified through the pattern of inflammatory molecules. These results imply that serum inflammatory molecule levels alone were insufficient data for identifying bacterial species. As different species of bacteria have common characteristics (such as lipopolysaccharide on gram-negative bacteria), a similar pattern of inflammatory molecules were produced by innate immune system (32). However, in order to identify bacterial species on the basis of the pattern of serum molecule expression levels, the present findings suggest that the detection of more molecules is necessary.

In the present study, IP-10 level in the viral infection group was found to be higher than in the bacterial infection group. Previous studies have indicated that IP-10 level is associated with viral infection, including hepatitis C virus (HCV) infection and HCV-human immunodeficiency virus co-infection (33-35). Dengue virus infection is associated with higher concentrations of IP-10 in patient serum compared with healthy individuals (36). Furthermore, IP-10 is induced by viral and bacterial infection. Higher levels of IP-10 may be detected in sepsis, severe sepsis and septic shock groups compared with systemic inflammatory response syndrome (37). High levels of IP-10 have previously been detected in preterm infants with bacterial infection (12). Although bacterial and viral infection induce IP-10 level, the highest levels of IP-10 in the present study were observed among influenza virus, culture-negative and culture-positive groups (data not shown). IP-10 is a ligand for CXCR3 chemokine receptor, and expression of IP-10 recruits CXCR3 expressing NK and T cells (38). CXCR3 and IP-10 defective mice exhibited a lower survival rate compared with wild type mice in a dengue infection model (39). The CXCR3-IP-10 pathway may be essential for clearance of dengue virus, which may explain why dengue virus induced the highest level of IP-10 expression in the present study.

In summary, bacterial infection was associated with the production of IL-6, IL-8 and IP-10. In addition, an increased IL-8 level was associated with gram-negative bacteria and a high IP-10 level was associated with dengue virus infection. Although the pattern of inflammatory molecules alone could not be used to identify bacteria species, these molecules may provide useful information for diagnosis and clinical treatment.

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