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A single-tube two-color flow cytometric method for distinguishing between febrile bacterial and viral infections

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ABSTRACT

The aim of this study was to develop a rapid single-tube two-color flow cytometric method to distinguish between febrile bacterial and viral infections.

In this prospective comparative study, the quantitative flow cytometric analysis of CD35 and CD64 on isolated human leukocytes was obtained from 286 hospitalized febrile patients, of which 197 patients were found to have either a bacterial (n = 136) or viral (n = 61) infection. The data from infected patients was compared to 49 healthy controls and 23 patients in an inflammatory state.

We developed a flow cytometric marker for bacterial infections, defined as the two-color bacterial infection index (TC-BI-index), by incorporating the quantitative analysis of CD35 and CD64 on isolated neutrophils, monocytes, and B-lymphocytes, which displayed 90% sensitivity and specificity in distinguishing between microbiologically confirmed bacterial (n = 77) and viral infections (n = 61) within 45 min.

We propose that TC-BI-index test will be useful in assisting physicians to ascertain whether antibiotic treatment is required, thus limiting unnecessary antimicrobial usage.

1. Introduction

Antibiotics, one of the cornerstones of the modern medicine, are intended for use in the treatment of illnesses caused by bacteria. Even the smallest doubt regarding a bacterial infection can lead a clinician to prescribe empiric antibiotic treatment just to be on the safe side, to eliminate the risk of a life-threatening infection. However, in addition to being ineffective and contributing to the development of toxicity and allergic reactions, the inappropriate treatment of viral illnesses or non-infective causes of inflammation with antibiotics may also contributes to the development of antibiotic resistance (French, 2005, Livermore, 2005), which is one of the most urgent public health problems on a global scale (Spellberg et al., 2008).

A major factor underlying the unnecessary use of antibiotics is the lack of rapid and accurate diagnostic tests. As previously reviewed, several methods have been developed over the decades to help the clinician to decide whether the infection is bacterial or viral in origin (Nuutila and Lilius, 2007, ten Oever et al., 2016). Many of them, like the erythrocyte sedimentation rate, serum CRP and cytokine levels, and leukocyte counts have relatively poor sensitivity and specificity, providing only supportive evidence towards confirming a diagnosis (Fischer et al., 2002, Korppi et al., 1997). Blood culturing, which has generally been used as a golden standard method in detecting bacterial infections, can be fairly laborious, time-consuming, and often negative in patients receiving antibiotics (Grace et al., 2001).

Among the more recent diagnostic methods, the serum procalcitonin level is a potential marker of bacterial infection in critically ill patients (Dubos et al., 2006), but appears to be correlated more to the severity of the infection (Pecile et al., 2004), particularly sepsis (van Rossum et al., 2004), rather than being an unequivocal marker of bacterial infection.

The high sensitivity of the PCR technique is also a significant negative aspect of that method. As an example, the viral genome is inevitably released from infected tissue weeks after the resolution of infection, often leading to a false positive result (Yang and Rothman, 2004). False negative PCR results can also occur due to gene polymorphism or when the target copy number is low, for example (Bacich et al., 2011, Jaton et al., 2010).

Clearly, there is an ongoing need for new sensitive and specific markers of bacterial infection. One candidate is the flow cytometric determination of host biomarkers, i.e. receptors, on the surface of blood leukocytes. The hypothesis behind the idea is that bacterial and viral

Abbreviations: TC-BI-index, two-color bacterial infection index

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Table 1
TC-BI-index variables CD35 RATIO, CD64 RATIO, NeuRATIO, MonRATIO, and the TC-BI-index itself in healthy controls (n = 49), in microbiologically confirmed viral (n = 61) and bacterial (n = 77) infections, and in clinically diagnosed bacterial infection (n = 59) expressed as mean (S.D.)

Variable (V)	Healthy control	Confirmed viral infection	Confirmed bacterial infection	Clinical bacterial infection	p-Values Tukey's test				
	(A; n = 49)	(B; n = 61)	(C; n = 77)	(D; n = 59)	ANOVA	A vs B	A vs C	B vs C	C vs D
CD35RATIO	0.497 (0.207)	0.703 (0.617)	1.596 (0.886)	1.620 (1.182)	$p < 0.001$	0.565	$p < 0.001$	$p < 0.001$	0.998
CD64RATIO	0.084 (0.020)	0.184 (0.119)	0.356 (0.199)	0.344 (0.163)	$p < 0.001$	0.003	$p < 0.001$	$p < 0.001$	0.970
*Neu*RATIO	0.506 (0.088)	0.469 (0.190)	0.654 (0.146)	0.663 (0.127)	$p < 0.001$	0.548	$p < 0.001$	$p < 0.001$	0.984
*Mon*RATIO	0.694 (0.085)	0.484 (0.270)	0.699 (0.158)	0.726 (0.148)	$p < 0.001$	$p < 0.001$	0.999	$p < 0.001$	0.821
TC-BI-index	0.007 (0.007)	0.010 (0.021)	0.153 (0.198)	0.146 (0.133)	$p < 0.001$	1.000	$p < 0.001$	$p < 0.001$	0.990

P-values for overall group differences tested using ANOVA. Group comparisons after ANOVA carried out using Tukey's multiple comparison technique.

infections induce different systemic profiles of proinflammatory cytokines (Slaats et al., 2016), which can lead to different expression patterns of certain cell surface infection markers on blood leukocytes in these different infection types. Flow cytometric bacterial infection markers offer significant benefits. Firstly, flow cytometry is a universally accepted instrument for detecting cell surface markers and is now available in the clinical laboratories of most hospitals. Compact, inexpensive, and easy-to-use benchtop flow cytometers lower the threshold to acquire the device even in small health care centers. Second, a multi-color staining technique enables the simultaneous determination of the expression of several receptors on leukocytes, even in a single test tube format. Yet another advantage is that the time window of quantitative receptor analysis from procuring blood samples to data handling and diagnosis is less than one hour.

Until now, increased expression of Fc-gamma-receptor I (FcγRI/CD64) on neutrophils has been the most widely used flow cytometric markers of infection (both bacterial and viral) (Nuutila, 2010), as well as the severity of sepsis (Hsu et al., 2011, ten Oever et al., 2016, Jamsa et al., 2015). However, while the presence of CD64 on neutrophils is a sensitive marker of bacterial infection, this marker is also highly expressed in DNA virus infections and thus cannot be used unambiguously in distinguishing between bacterial and viral diseases (Nuutila et al., 2008).

Compared with neutrophil CD64, neutrophil complement receptor1 (CR1/CD35) seems to be a more sensitive and specific bacterial infection marker. We have discovered in our previous study that the average expression level of CD35 on neutrophils in bacterial infections was over three-fold higher than in viral infections and in healthy controls, displaying 85% sensitivity and specificity in distinguishing between bacterial and viral infections (Nuutila et al., 2006). We also discovered in that particular study that any single bacterial infection marker alone cannot be used to reliably differentiate between bacterial and viral infections and that diagnostic accuracy can be improved by a combination of several (3–4) bacterial infection markers.

This fundamental theory of combination was afterwards confirmed in our previous reports presenting first two genuine multiparametric flow cytometric bacterial infection markers, namely the Bacterial Infection Score (BIS) method and Bacterial Infection (BI)-INDEX, where CD35 data was incorporated with CD32, CD88, and MHC class I data and with CD10 and CD282 data, respectively. Both of these computational flow cytometric markers displayed at least 90% sensitivity and specificity in distinguishing between microbiologically confirmed bacterial and viral infections within 45 min (Nuutila et al., 2014, 2013).

The aim of the study was to update the present three-parameter BI-INDEX method and find out the minimum amount of CD antigens (leukocyte cell surface receptors) that need to be determined to be able to calculate the effective bacterial infection index for bacterial vs. viral differentiation. In this study, we present a novel two-color (TC) one-tube flow cytometric bacterial infection (BI) index (TC-BI-index), incorporating data on CD35 and CD64 expression from isolated blood leukocytes and distinguishing between bacterial and viral infections with 90% sensitivity and specificity.

We propose that the rapid TC-BI-index test can assist physicians in deciding whether antibiotic treatment is necessary, thus reducing unnecessary antimicrobial use.

2. Materials and methods

2.1. Study subjects

The local Ethical Committee of Turku University/Turku University Hospital approved the study protocol.

Heparin-anticoagulated blood samples for quantitative receptor analysis were obtained prospectively from 286 hospitalized febrile patients with suspected infection and 49 non-hospitalized healthy volunteer controls (26 women/23 men; age: 34 ± 13 years (mean ± SD)). Fever (measured in the hospital) was regarded as a body temperature of at least 37.5 °C. Patients receiving corticosteroids or other immunosuppressive agents or who were hospitalized for over 48 h before procuring blood samples were excluded from the study. Other exclusion criteria included patients with immunosuppression (e.g., HIV infection, hematological malignancy or chemotherapy).

At the time of admission, antibacterial or antiviral treatment was initiated in 83% of patients with confirmed bacterial or viral infection. An underlying disease, most often cardiovascular disease, was present in 65% of bacterial and 22% of viral infection cases (supplementary data, Table 1).

In total, 197 out of 286 patients were diagnosed with either a bacterial (n = 136) or viral (n = 61) infection (Fig. 1). The bacterial infection group included cases that were microbiologically confirmed (n = 77) or clinically diagnosed (n = 59). All virus infection cases were confirmed. For the purpose of this study, we attempted to confirm all the diagnoses either microbiologically or serologically using “gold standard” methods, but also accepted clinical diagnoses. Clinically diagnosed bacterial infections (n = 59) were classified by the attending physician based on signs and symptoms, in combination with the clinical course of the disease.

Bacterial culture was used as the gold standard for the diagnosis of bacterial infections. Definitions of confirmed bacterial infections included isolation of an organism by culture from the blood (sepsis), urine levels $> 10^5$/mL in patients displaying clinical symptoms and signs of pyelonephritis, needle aspiration of abscess or empyema, stool samples of patients with gastroenteritis symptoms, or sputum samples and chest radiographs of patients with community-acquired pneumonia. Apart from bacterial culture, diagnosis of Lyme borreliosis was based on clinical examination and positive serological tests (detection of *Borrelia*-specific antibodies with ELISA).

Serological tests and PCR served as the gold standards for the diagnosis of viral infections. Diagnosis of a confirmed viral infection required the detection of IgM antibodies or at least a 4-fold increase in IgG antibodies in the serum, and detection of viral antigens in serum, cerebrospinal fluid, or blister (Varicella zoster virus). Nucleic acids