

## PATHOGEN-SPECIFIC ANTIBODY PROFILES IN PATIENTS WITH SEVERE SYSTEMIC INFECTIONS

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### Abstract

Infections are often caused by pathobionts, endogenous bacteria that belong to the microbiota. Trauma and surgical intervention can allow bacteria to overcome host defences, ultimately leading to sepsis if left untreated. One of the main defence strategies of the immune system is the production of highly specific antibodies. In the present proof-of-concept study, plasma antibodies against 9 major pathogens were measured in sepsis patients, as an example of severe systemic infections. The binding of plasma antibodies to bacterial extracellular proteins was quantified using a semi-automated immunoblot assay. Comparison of the pathogen-specific antibody levels before and after infection showed an increase in plasma IgG in 20 out of 37 tested patients. This host-directed approach extended the results of pathogen-oriented microbiological and PCR diagnostics: a specific antibody response to additional bacteria was frequently observed, indicating unrecognised polymicrobial invasion. This might explain some cases of failed, seemingly targeted antibiotic treatment.

**Keywords:** Systemic infection, sepsis, pathogen-specific IgG, serology, antibody response.

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### Introduction

Bacteria, commensals as well as pathogens, are constantly surrounding the human body, frequently as members of the microbiota. The innate and adaptive immune systems are responsible for preventing these bacteria from penetrating the protective barrier of skin and mucous membranes, e.g. through the production of specific (neutralising) antibodies (Byrd *et al.*, 2018). Favoured by trauma and invasive surgical procedures, these endogenous opportunistic

pathogens, also referred to as pathobionts, can occasionally cause severe infections, ranging from bone and soft tissue infections to implant-associated infections, up to sepsis (Benito *et al.*, 2019). Sepsis, defined as a 'life-threatening organ dysfunction caused by a dysregulated host response to infection' (Singer *et al.*, 2016), is a leading cause of death (Fleischmann *et al.*, 2015; La Suarez De Rica *et al.*, 2016). Sepsis caused by *Staphylococcus aureus* (*S. aureus*) induces a strain-specific IgG response. An increase in anti-*S. aureus* antibodies in patient

sera during an infection is observed in most patients, often in the early stages of the disease (Kolata *et al.*, 2011). This suggests that studying the adaptive immune system's reaction to bacterial invasion could shed light on the infection process, complementing pathogen-directed techniques such as microbiological culture and PCR methods.

Positive microbial culture provides clinicians with information about the identity as well as antibiotic susceptibility of the causative bacterial species and, hence, guides a subsequent antibiotic treatment that is lifesaving (Dellinger *et al.*, 2013; Kim and Park, 2019). However, up to 70 % of all clinically diagnosed sepsis cases are culture-negative (Cohen *et al.*, 2015), showing that standard microbiological culture lacks sensitivity. Moreover, standard microbiological culture is vulnerable to antibiotic treatment and sampling error (false positive or false negative), especially in the setting of poly-microbial infections. On the other hand, sequence-based pathogen detection by PCR allows for faster and more sensitive pathogen diagnostics (Trung *et al.*, 2019) despite being often unable to determine whether the pathogen is actually responsible for the infection, leading to false positives (poor specificity) (Yamamoto, 2002).

Pathogens that induce an adaptive immune response are probably relevant in the infection process and may be identified by measuring pathogen-specific IgGs in the blood. Such a host-directed approach would be robust against antibiotic therapy. However, the interpretation of the antibody levels is complicated by the fact that many sepsis-causing agents are members of the human microbiota. Therefore, the individual history of exposure to these pathogens results in highly variable basal pathogen-specific plasma antibody levels, precluding the definition of a "diagnostic threshold" (Hornef, 2015). Studies have previously shown that IgG titers against certain *S. aureus* proteins have a diagnostic value in different clinical settings, including osteomyelitis, diabetic foot infections, epidermolysis bullosa and prosthetic joint infections (PJI) (Marmor *et al.*, 2016; Nishitani *et al.*, 2015; Oh *et al.*, 2018; van der Kooi-Pol *et al.*, 2013). Moreover, high basal anti-*S. aureus* antibody titers show protection against severe *S. aureus* infections/complications (Fritz *et al.*, 2013; Michalik *et al.*, 2020; Stentzel *et al.*, 2015). However, little is known about the diagnostic potential of the antibody response against other frequent nosocomial pathogens.

The measurement of increases in specific antibody levels during infection, as they are consistently observed in patients with *S. aureus* bacteraemia (Kolata *et al.*, 2011), could also be of value in infections caused by other bacterial pathogens. The present proof-of-concept study demonstrated that sepsis patients developed an IgG response, which was specific for the invasive pathogen(s). This approach might complement pathogen-directed diagnosis, especially of secondary and poly-microbial infections.

## Materials and Methods

### Study design and patient material

Within a prospective clinical trial, 52 sepsis patients, fulfilling the 2001 consensus definition of severe sepsis or septic shock, were recruited at the University Medicine Greifswald between 2011 and 2012 (Levy *et al.*, 2003). The study was approved by the institutional ethics committee of the University Medicine Greifswald (VYOO Amendment, BB138/10a). All patients provided written informed consent for study participation. Around 71 % of those sepsis patients ( $n = 37$ ; 24 male and 13 female; age range: 28-85 years; median: 72 years) had a standard microbiological diagnosis based on blood cultures ( $n = 17$ ) or respective sites of infection ( $n = 20$ ) and were, thus, included in the study (for further information on the patient cohort consult Table 1). Results of a multiplex PCR test (VYOO) for the detection of pathogens in blood taken at sepsis diagnosis were available for 16 of the 37 patients investigated in the study (Sachse *et al.*, 2009). Among those 16 patients, 81 % (13/16) showed concordance with the microbiological findings. Moreover, poly-microbial infections were detected in 35 % and 24 % of the patients, by microbiology and PCR, respectively. 23 of the 37 patients (62.2 %) presented with serum lactate levels  $> 2$  mmol/L, fulfilling the sepsis-3 definition criterion for septic shock (Singer *et al.*, 2016). For each patient, two plasma samples were analysed, with a median time span ( $\Delta T$ ) of 16 d. For the earlier time point, blood was obtained either at the time of clinical diagnosis of sepsis ( $n = 37$ ) or, whenever possible, at a time point before sepsis diagnosis ( $n = 35$ ). This was possible because diagnostic samples are routinely stored for 6 d at the Institute of Clinical Chemistry and Laboratory Medicine, University Medicine Greifswald, Greifswald, Germany. These reserved diagnostic samples were made available for the present study and used to determine basal plasma antibody levels. The second patient sample was taken at the latest possible time point during infection. Two plasma samples from each of 8 healthy control subjects [5 male (62.5 %) and 3 female (37.5 %)], with a median  $\Delta T$  of 20 d, were likewise analysed.

### Bacterial cultivation and protein extraction

The bacterial strains isolated from positive blood cultures or other samples taken from sites of infection were stored as glycerol stocks at  $- 80$  °C. For the antigen preparation, overnight cultures of the respective bacterial strains in tryptic soy broth (TBS, Oxoid Ltd, Cheshire, UK) were used to inoculate the main culture to an optical density (OD) at 595 nm of 0.05. The cultures were grown at 37 °C and 100 shakes per min (spm) for a total of 8 h. The culture supernatants were harvested and the secreted proteins extracted as previously described (Holtfreter *et al.*, 2009). Briefly, the culture supernatant was filtered through a 0.2  $\mu$ m membran (Merck).

**Table 1. Baseline characteristics of the sepsis patient cohort included in the study ( $n = 37$ ).** <sup>1</sup> m, male; f, female. <sup>2</sup> B, primary bacteraemia; B/ST, bone and soft tissue infection; C, catheter-associated infection; IA, intraabdominal infection; P, pneumonia; U, urinary tract infection. <sup>3</sup> Septic shock was defined according to the Sepsis-3 definition by serum lactate levels > 2 mmol/L (Singer *et al.*, 2016). <sup>4</sup> 90-d survival. <sup>5</sup> A, abscess; C, catheter; DF, drain fluid; N, nose; P, perineum; T, tracheal secretion; W, wound. \* *E. faecium*; *E. faecalis*. <sup>†</sup> for two patients no serum lactate levels were available. CoNS: coagulase-negative staphylococci; nd: not determined.

Patient characteristics						Microbiological findings		PCR results
ID	Age	Sex <sup>1</sup>	Infection focus <sup>2</sup>	Diagnosis <sup>3</sup>	Survival <sup>4</sup>	Blood culture	Other material <sup>5</sup>	VYOO®
1	58	m	IA	sepsis <sup>†</sup>	yes	<i>S. haemolyticus</i>	Enterococci <sup>W,*</sup> ; <i>C. albicans</i> <sup>W</sup>	<i>B. fragilis</i> ; CoNS; <i>E. faecium</i>
2	55	m	IA	sepsis	no		<i>E. faecium</i> <sup>W</sup>	CoNS; <i>E. faecium</i>
3	76	m	nd	septic shock	no	<i>S. aureus</i>	<i>S. aureus</i> <sup>T</sup>	<i>S. aureus</i>
7	84	f	IA	septic shock	no		<i>E. faecium</i> <sup>W</sup>	<i>E. faecium</i>
8	65	m	B/ST	septic shock	no		<i>S. epidermidis</i> <sup>W</sup> ; CoNS <sup>T</sup> ; <i>Candida species</i> <sup>T</sup>	<i>S. aureus</i>
10	81	f	P	septic shock	no	<i>E. coli</i>	<i>C. albicans</i> <sup>N,P</sup>	<i>E. coli</i>
11	72	m	IA	sepsis	yes		<i>S. aureus</i> <sup>N,P</sup>	
12	50	m	IA	septic shock	yes		<i>E. faecalis</i> <sup>W</sup> ; <i>C. albicans</i> <sup>W</sup>	
13	57	f	nd	septic shock	yes	<i>S. hominis</i>		
14	52	f	IA	sepsis	nd		<i>E. coli</i> <sup>P,DF</sup>	
15	40	f	P	septic shock	yes	<i>S. pneumoniae</i> ; <i>S. hominis</i>		
16	28	m	IA	sepsis	nd	<i>S. haemolyticus</i> ; <i>S. epidermidis</i>	<i>S. aureus</i> <sup>W</sup>	
17	78	f	IA	septic shock	yes		<i>E. faecium</i> <sup>A</sup>	
18	72	f	P	sepsis	nd	<i>C. glabrata</i> ; <i>S. epidermidis</i>	<i>A. hydrophila/caviae</i> <sup>W</sup>	
21	60	m	IA	septic shock	no		<i>E. coli</i> <sup>W</sup>	<i>E. coli</i> ; CoNS
22	56	m	IA	sepsis	nd	<i>S. haemolyticus</i> ; <i>E. faecalis</i>	<i>S. haemolyticus</i> <sup>C</sup> ; <i>E. faecalis</i> <sup>C</sup>	
24	78	m	IA	septic shock	nd		<i>K. pneumoniae</i> <sup>W</sup> ; <i>E. coli</i> <sup>W</sup> ; <i>E. faecalis</i> <sup>W</sup>	
25	85	f	P	septic shock	no		Enterococci <sup>W,*</sup>	
26	77	m	B/ST	sepsis	yes	<i>S. schleiferi</i>	<i>S. aureus</i> <sup>N</sup>	<i>S. aureus</i>
27	73	m	IA	septic shock	yes	<i>E. faecalis</i>		<i>E. coli</i> ; CoNS; <i>E. faecium</i>
28	77	m	B	septic shock	yes	<i>E. faecalis</i> ; <i>S. epidermidis</i>		
29	81	m	IA	septic shock	nd		<i>E. coli</i> <sup>T</sup>	<i>E. coli</i> ; CoNS
31	68	m	P	sepsis	yes		Streptococci <sup>T</sup> ; CoNS <sup>T</sup> ; <i>E. coli</i> <sup>T</sup>	
32	51	m	IA	septic shock	nd	<i>K. pneumoniae</i>		
34	78	f	B/ST	septic shock	no		<i>S. aureus</i> <sup>W</sup>	
35	75	m	nd	sepsis	yes		<i>K. pneumoniae</i> <sup>C</sup>	
36	44	m	nd	sepsis <sup>†</sup>	nd	<i>S. marcescens</i>		
41	49	m	IA	septic shock	yes		<i>E. faecium</i> <sup>W,T,DF</sup>	<i>B. fragilis</i> ; <i>E. faecium</i> ; <i>P. mirabilis</i>
43	76	f	P	sepsis	nd		<i>K. oxytoca</i> <sup>T,C</sup>	<i>S. aureus</i>
45	46	f	IA	septic shock	yes		<i>P. mirabilis</i> <sup>W</sup> ; Enterococci <sup>W</sup>	<i>E. coli</i> ; <i>E. faecium</i> ; <i>K. oxytoca</i>
46	68	m	nd	septic shock	no	<i>P. aeruginosa</i>	<i>P. aeruginosa</i> <sup>T</sup>	<i>B. fragilis</i> ; <i>P. aeruginosa</i>
47	54	m	U	septic shock	yes		Enterococci <sup>T,C,*</sup>	
48	75	m	B/ST	sepsis	yes	<i>S. aureus</i>	<i>S. aureus</i> <sup>W</sup>	<i>S. aureus</i>
50	81	f	IA	septic shock	nd		Enterococci <sup>W,*</sup> <i>P. mirabilis</i> <sup>W</sup>	Enterococci*; <i>P. mirabilis</i> ; <i>C. glabrata</i>
51	81	m	IA	sepsis	yes	<i>S. epidermidis</i>	<i>E. faecium</i> <sup>W</sup> ; <i>C. freundii</i> <sup>W</sup> ; <i>Bacteroides spp.</i> <sup>W</sup> ; <i>C. albicans</i> <sup>W</sup>	
53	80	m	IA	septic shock	yes		<i>P. aeruginosa</i> <sup>C</sup>	
54	70	f	IA	septic shock	yes	<i>S. haemolyticus</i> ; <i>E. coli</i>		

**Table 2. Settings for immunoblot assays.** Group 1 bacteria are the most common sepsis pathogens. Group 2 bacteria were used for a personalised approach in selected cases.

Sample	Dilution	
Exoproteomes of the following bacterial species (final concentration: 1 mg/mL)	Primary antibody (plasma)	Detection antibody (anti-human IgG POD)
<b>Group 1</b>		1 : 1,000
<i>Enterococcus faecium</i>	1 : 2,000	
<i>Escherichia coli</i>	1 : 2,000	
<i>Serratia marcescens</i>	1 : 2,000	
<i>Pseudomonas aeruginosa</i>	1 : 2,000	
<i>Klebsiella pneumoniae</i>	1 : 2,000 1 : 1,000	
<i>Streptococcus pneumoniae</i>	1 : 2,000	
<i>Staphylococcus aureus</i>	1 : 10,000	
<i>Staphylococcus epidermidis</i>	1 : 4,000	
<i>Staphylococcus haemolyticus</i>	1 : 4,000	
<b>Group 2</b>		
<i>Staphylococcus hominis</i>	1 : 1,000	
<i>Enterococcus faecalis</i>	1 : 1,000 1 : 2,000	
<i>Proteus mirabilis</i>	1 : 2,000 1 : 1,000	
<i>Klebsiella oxytoca</i>	1 : 1,000	
<i>Tetanus toxoid</i> (final concentration: 0.2 mg/mL)	1 : 4,000	

The secreted proteins were precipitated using trichloroacetic acid (TCA, Roth; final concentration 10 %), washed with 70 % ethanol and resolved in rehydration buffer containing 8 M urea (Merck), 2 M thiourea (Merck) and 2 % 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulphonate (CHAPS; Merck). Protein concentrations were measured using the Bradford Protein Assay (Bio-Rad) (Bradford, 1976). The purified protein samples were stored at  $-80^{\circ}\text{C}$ .

For *S. aureus*, the protein-A-deficient mutant of strain USA300 (*S. aureus* USA300 $\Delta$ *spa*; Stentzel *et al.*, 2015) was used to prevent non-specific antibody binding. Moreover, staphylococci were cultured under iron limitation, namely in TSB supplemented with 2,2'-bipyridyl (Sigma-Aldrich; final concentration 600  $\mu\text{M}$ ), to enhance the secretion of immunogenic proteins (Stentzel *et al.*, 2014).

#### Semi-automated immunoblot assay

Antibody binding to extracellular proteins of common sepsis pathogens as well as tetanus toxoid (TT, Statens Serum Institute, Copenhagen, Denmark) was quantified using semi-automated quantitative immunoblotting (Peggy Sue™ Simple Western Assay; ProteinSimple®, Santa Clara, CA, USA), which uses capillary electrophoresis for antigen separation followed by exposure to the antibody samples and quantification of antibody binding

by chemiluminescence. Two plasma samples per study subject were analysed, one obtained at the earliest and one at the latest available time point. Antigen-specific plasma antibodies were measured according to the Peggy Sue™ Simple Western Assay manual (Kit: 12-230 kDa Peggy Sue™ or Sally Sue™ Separation Module SM-S001 and DM-003) using 1 mg/mL and 0.2 mg/mL of extracellular proteins and TT, respectively. In brief, bacterial proteins were mixed with 5 $\times$  mastermix containing sample buffer, DTT and a fluorescent standard. Protein samples and biotinylated molecular weight (MW) standards from ProteinSimple® were heated for 5 min at  $95^{\circ}\text{C}$ . Human plasma samples served as the source of primary antibodies and their dilution was optimised for each bacterial species (1 : 1,000 to 1 : 10,000) (Table 2). The polyclonal detection antibody [goat anti-human IgG (H+L) horse-radishperoxidase (POD)-conjugated; 109-035-088, Dianova, Hamburg, Germany] was used at a final concentration of 0.8 ng/mL. The different reagents, including protein samples, blocking reagent, diluted plasma samples, detection antibody, chemiluminescent substrate, separation and stacking matrices were loaded to designated wells in a 384-well plate. The plate was loaded into the instrument and separation as well as detection steps were performed automatically, analogous to standard immunoblotting procedures. The chemiluminescence signals generated upon reaction



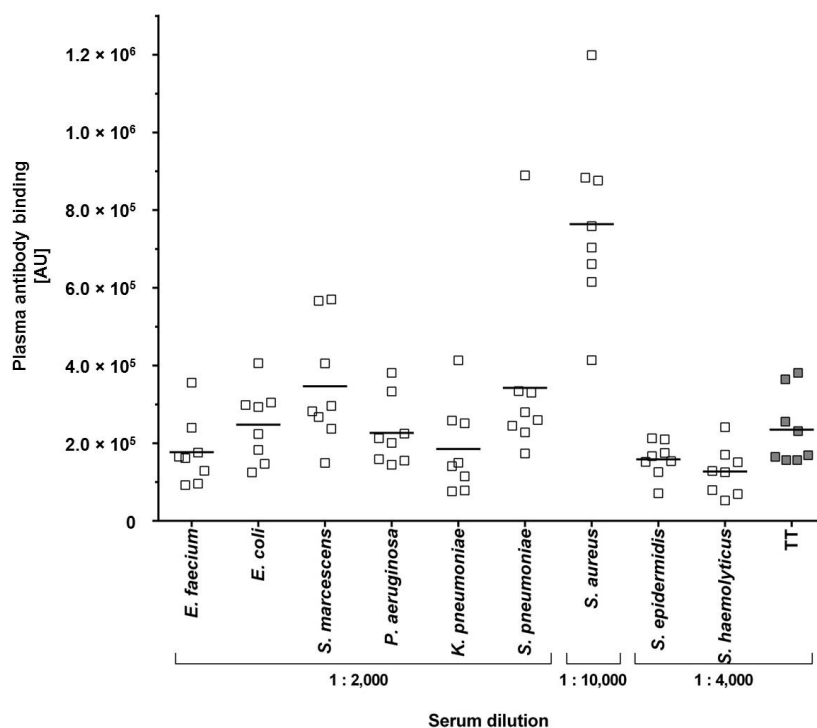
with a luminol/peroxide substrate solution were displayed as electropherograms and automatically analysed using Compass for SW software Version 3.1.7 (ProteinSimple®). The areas under the curve of manually set peaks (peak fit width, 2; baseline, 0; area calculation, Gaussian fit) were summed to obtain the total signal. Data were analysed and visualised using GraphPadPrism Version 6 (GraphPad Software). The increase in pathogen-specific IgG was determined by comparing plasma antibody binding during sepsis with that prior to infection. To exclude non-specific B cell activation in the study cohort, plasma antibody binding to the recall antigen TT was also measured at both time points.

### Statistical analysis

The statistical analysis was performed using GraphPad Prism Version 6. To compare changes in plasma IgG binding in healthy subjects and sepsis patients, the data were analysed using the unpaired *t*-test.  $p \leq 0.05$  was considered statistically significant.

## Results

Basal levels of pathogen-specific plasma IgG binding in healthy subjects showed substantial inter-individual and inter-species variations (Fig. 1). However, binding of plasma IgG to TT or to the extracellular proteins of the 9 most common sepsis-causing bacteria (listed in Table 2, group 1) did not change significantly over time (mean = 0.9-fold;  $\Delta T = 20$  d; Fig. 2). Therefore, the threshold for a significant rise in antibody binding was set, based on results from healthy subjects, to a 1.6-fold increase [mean + 3 × standard deviation (SD)].



**Fig. 1. Basal pathogen-specific plasma IgG levels in healthy subjects.** Plasma IgG binding to extracellular proteins of frequent sepsis pathogens was quantified using semi-automated quantitative immunoblotting. TT served as a control antigen (grey squares). Plasma samples were diluted between 1 : 2,000 and 1 : 10,000, as indicated, to ensure measurement within the linear range of the assay (Table 2). The area under the curve of manually set peaks was calculated using Compass SW Version 3.1.7. All signals were added to obtain the total signal of antibody binding (AU, arbitrary units). Squares show the mean values of two replicates; means are indicated.

When tested for increases in IgG binding to the microbiologically diagnosed infectious agents, the microbiological diagnosis was corroborated in 20 out of 37 sepsis patients (54 %) (Fig. 2,  $\Delta T = 16$  d). In contrast, the anti-TT antibody levels remained stable in most of the patients as already observed for the healthy control group. However, due to one outlier (patient 28), the mean concentration of plasma antibodies against the recall antigen TT increased slightly in the sepsis patient group (Fig. 2).

To assess the specificity of the anti-bacterial responses, patients showing an increase in pathogen-specific antibody binding were further analysed for their antibody responses to the 9 most frequently diagnosed bacterial species. 3 groups of patients could be distinguished (Fig. 3, Table 3).

- In 4 patients, IgG binding to a single pathogen, also identified in the blood culture, increased significantly (9- to 37-fold) and in two cases, patient 10 and 46, the result was additionally confirmed by PCR (Fig. 3a, Table 3). Together with the lack of significant changes in the anti-TT titers, this confirmed that the antibody responses were caused by the invasive bacteria.
- In 15 patients, most of whom suffered intra-abdominal infections, antibody responses to additional pathogens were observed. 5 of those patients exhibited only minor increases in IgG levels (1.6- to 5-fold). In 9 of the 15 patients, the strongest increase in plasma antibodies was directed against the microbiologically diagnosed pathogen (Fig. 3b, Table 3). In the remaining 6 cases bacteria other than the microbiologically diagnosed ones elicited a stronger IgG response (Fig. 3c, Table 3). In patient 41, for example, *E. faecium* was identified by both microbiology

and PCR, whereas the increase in IgG binding against *S. marcescens* exceeded the increase against *E. faecium* by more than 10-fold.

- In 1 patient, a pronounced increase in anti-TT antibodies exceeded the pathogen-specific responses; however, the minor increases against enterococci and *S. epidermidis* were still consistent with the microbiological findings (patient 28, Table 3).

In 21 of the 37 patients examined, no pathogen could be identified by PCR. Interestingly, 17 of these patients showed only minor increases in IgG levels ( $\leq 5$ -fold). Of the 16 patients with positive PCR results, the pathogen diagnosis result was concordant with the observed antibody increase in 9 cases (56 %). Similarly, the serological approach was often able to identify other or additional pathogens when compared to PCR. In 4 PCR-negative patients (patients 14, 15, 36 and 51, Table 1) a possible causative agent could be diagnosed with the serological approach used.

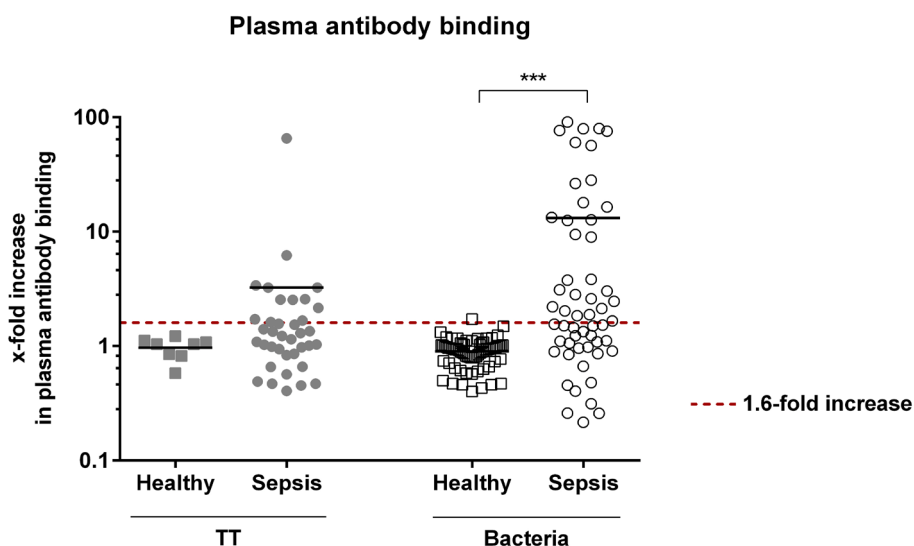
## Discussion

Serological assays are widely used in disease diagnostics, but typically employ (single/multiple) well-defined recombinant proteins to detect pathogen-specific antibodies. For instance, a study of patients suffering PJI used a selection of 16

recombinant proteins from 5 different bacterial species to determine serum IgG levels. Indeed, staphylococcal infections were identified with a sensitivity/specificity of 72.3 % and 80.7 %, respectively (Marmor *et al.*, 2016). In another study by Nishitani *et al.* (2015), where 14 protein candidates were tested in a patient cohort with *S. aureus* culture-confirmed musculoskeletal infections, the *S. aureus* antigens IsdB and Hla showed the highest diagnostic value. Nevertheless, the authors recommended the use of a larger set of antigens, allowing for a higher sensitivity as compared to a single antigen.

To establish a reliable diagnostic assay, it would be necessary to identify immunogenic proteins that are conserved within a species of interest, to ensure optimal sensitivity and specificity. However, nosocomial infections, including sepsis, are usually caused by a multitude of different bacterial species. Furthermore, the immune proteome of some of those pathogens is often still enigmatic. This has prompted the exploration in the present study of a novel approach, where complex protein extracts from common sepsis pathogens were used to determine IgG binding in sepsis patients as well as healthy subjects, using a semi-automated quantitative immunoblot assay.

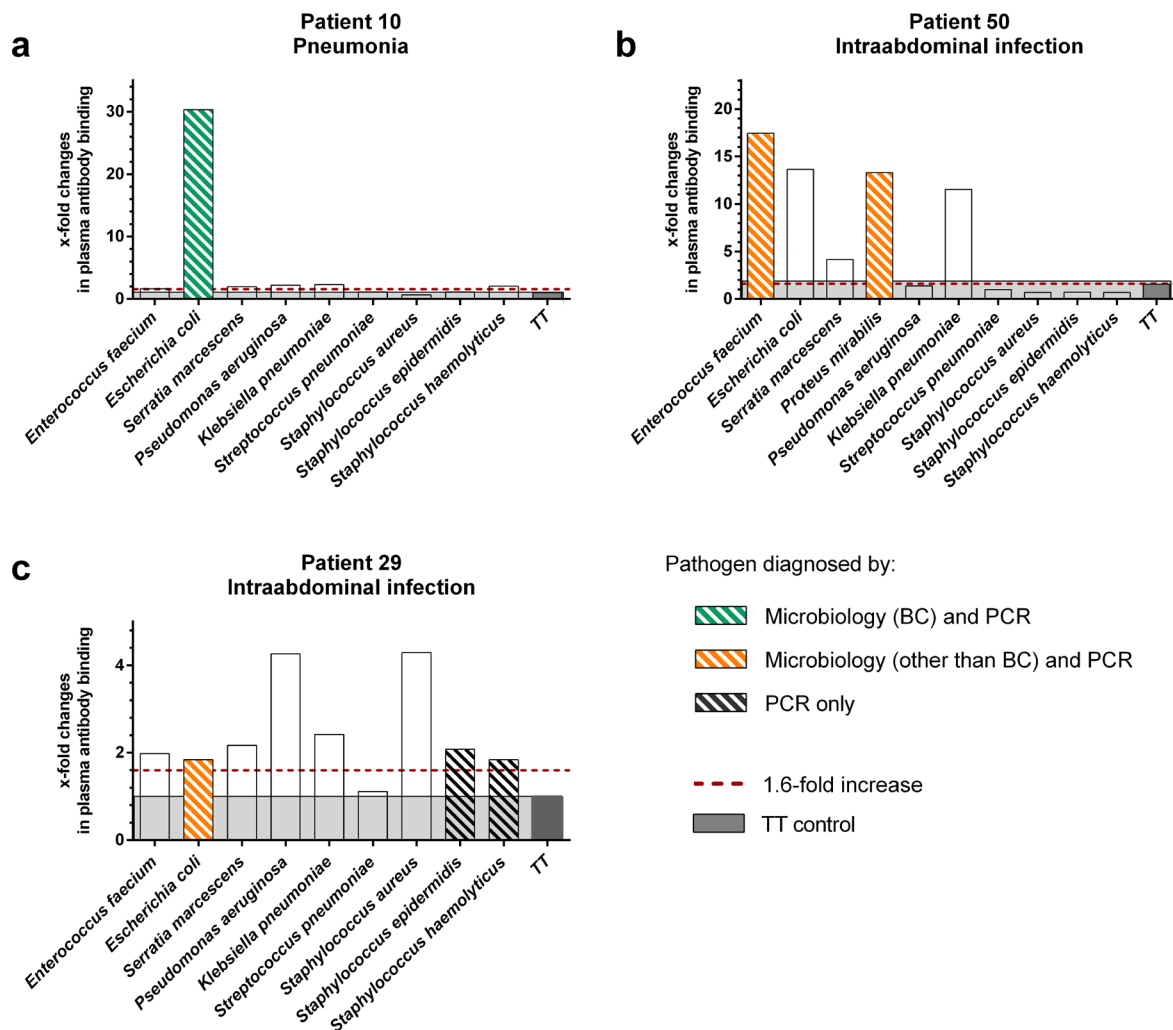
The humoral response to antigenic or inflammatory challenges, such as infection, vaccination or trauma, has been proposed to comprise the polyclonal activation of memory B cells of unrelated specificity (Bernasconi *et al.*, 2002). Remarkably, in the absence of



**Fig. 2. Antibody kinetics in healthy subjects and sepsis patients.** Plasma antibody binding to the recall antigen TT, as well as to extracellular proteins of frequent sepsis pathogens, was quantified using semi-automated quantitative immunoblotting. In patients, plasma antibody binding during infection was compared with that prior to the clinical diagnosis of sepsis using routinely reserved diagnostic samples. In healthy subjects, a comparable time period was investigated. In healthy subjects, the plasma antibodies specific for all tested antigens remained stable over time ( $\Delta T = 20$  d). The same was observed for the TT-specific antibody levels in sepsis patients ( $\Delta T = 16$  d). In contrast, many sepsis patients showed a significant increase in plasma antibody binding directed against the diagnosed pathogen. Red lines mark the threshold of increase (mean +  $3 \times$  SD; values of healthy subjects). Single squares (healthy subjects) and dots (sepsis patients) show the means of two replicate values; means are indicated. Healthy subjects and sepsis patients were compared using the unpaired *t*-test. \*\*\*  $p < 0.001$ .

exposure to the recall antigen TT, a moderate increase in anti-TT IgG was found in patients following cardiothoracic surgery (Pötschke *et al.*, 2012). Hence, to account for a possible non-specific B cell activation

in the study patient cohort, the anti-TT IgG response was also monitored. At first, IgG binding to 9 common sepsis pathogens (Table 2, group 1) was measured in the healthy control group (Fig. 1) and basal antibody



**Fig. 3. Pathogen-specific antibody responses in sepsis patients.** Plasma antibody binding to extracellular proteins of frequent sepsis pathogens was quantified using semi-automated quantitative immunoblotting. TT served as a control antigen. Plasma antibody binding during infection was compared with that prior to clinical diagnosis of sepsis. In patients showing an increase in plasma antibody binding directed against the diagnosed pathogen, plasma antibody binding to all 9 sepsis pathogens was determined. 3 typical cases are shown, where serology was consistent with the microbiological culture and PCR. (a) IgG binding was selectively increased to a single pathogen; (b) additional pathogens were identified by serology; (c) the strongest IgG response was caused by bacteria other than the microbiologically diagnosed ones. Red lines mark the threshold of increase (mean + 3× SD; values of healthy subjects, Fig. 2). Bars represent individual values. Colours indicate whether pathogens were diagnosed by microbiological culture [green, blood culture (BC); orange, other microbiological culture]; colour in combination with hatching indicates concordance of microbiological and PCR results; the grey area shows the response to the control antigen TT.

**Table 3. Heatmap to visualise the increases in pathogen-specific and TT-specific antibody levels in the sera of all 20 sepsis patients who showed an increase.** For each patient, the two time points of plasma sampling, at which the pathogen-specific IgG binding was determined, are shown. Furthermore, the increase in IgG response, the microbiological diagnosis and the focus of infection are listed. The patients are grouped according to the 3 typical cases mentioned in the text and shown in Fig. 3a-c. ND, not determined. 1ECOL (*E. coli*); ENTFA (*E. faecalis*); ENTFC (*E. faecium*); KLEO (*K. oxytoca*); KLEP (*K. pneumoniae*); PROM (*P. mirabilis*); PSEA (*P. aeruginosa*); SERM (*S. marcescens*); STAA (*S. aureus*); STAE (*S. epidermidis*); STAHA (*S. haemolyticus*); STAHO (*S. hominis*); STRP (*S. pneumoniae*). <sup>2</sup>B, primary bacteraemia; B/ST, bone and soft tissue infection; IA, intraabdominal infection; P, pneumonia.

Group	Patient ID	Day of sampling	ENTFC	ENTFA	ECOL	SERM	PROM	PSEA	KLEP	KLEO	STRP	STAA	STAE	STAHA	STAHO	TT	Microbiological diagnosis	Focus of infection?
i)	10	-2 14	1.6	ND	30.3	2.0	ND	2.2	2.3	ND	1.1	0.7	1.2	2.1	ND	1.0	ECOL	P
	15	0 14	2.2	ND	2.7	1.3	ND	1.0	2.4	ND	17.9	2.1	1.2	1.7	3.8	1.7	STRP, STAHO	P
	36	-3 14	1.1	ND	2.2	36.9	ND	1.2	0.9	ND	1.0	0.9	1.0	1.0	ND	0.9	SERM	ND
	46	-1 14	1.0	ND	1.3	1.6	ND	9.0	2.0	ND	0.7	1.9	1.3	0.9	ND	1.0	PSEA	ND
	1	-1 15	44.3	ND	3.3	3.0	ND	12.7	4.1	ND	7.9	13.3	38.4	56.8	ND	6.2	ENTFC, STAHA	IA
	2	-5 14	16.4	ND	3.5	2.2	ND	4.3	2.9	ND	3.1	1.3	8.0	8.0	ND	2.2	ENTFC	IA
	14	-6 10	5.4	ND	53.9	3.3	ND	51.3	8.9	ND	4.0	1.3	2.4	1.5	ND	2.6	ECOL	IA
	24	-3 14	2.1	1.5	2.5	1.7	ND	2.2	3.8	ND	1.0	0.9	2.1	1.3	ND	1.3	KLEP, ECOL, ENTFA	IA
	31	-6 14	0.5	ND	1.1	1.2	ND	1.7	1.3	ND	1.5	1.5	1.1	2.1	ND	1.2	ECOL, CNS, STRP	P
	43	-2 14	6.8	ND	7.6	9.9	ND	8.8	5.0	26.4	4.3	2.7	9.2	18.4	ND	3.3	KLEO	P
ii)	45	-3 14	47.4	ND	5.9	1.7	2.8	1.2	6.1	ND	1.8	3.0	2.2	2.6	ND	1.4	ENTFC, PROM	IA
	48	-2 14	3.6	ND	1.4	2.8	ND	1.5	1.1	ND	1.5	12.5	5.8	4.3	ND	0.9	STAA	B/ST
	50	-3 14	17.5	ND	13.7	4.2	13.3	1.4	11.5	ND	1.0	0.7	0.7	0.7	ND	1.6	ENTFC, PROM	IA
	16	-2 14	3.1	ND	2.8	3.4	ND	3.7	3.7	ND	1.7	1.5	3.1	2.6	ND	3.4	STAA, CNS	IA
	21	-1 14	5.9	ND	9.5	7.3	ND	39.9	4.5	ND	3.6	1.2	2.0	1.1	ND	3.3	ECOL	IA
	29	-2 14	2.0	ND	1.8	2.2	ND	4.3	2.4	ND	1.1	4.3	2.1	1.8	ND	1.0	ECOL	IA
	35	-1 14	3.3	ND	1.5	1.9	ND	2.0	1.6	ND	1.4	1.3	2.3	2.0	ND	1.1	KLEP	ND
	41	-1 14	28.2	ND	51.7	344.4	ND	3.3	14.4	ND	5.9	2.0	1.2	2.4	ND	2.5	ENTFC	IA
	51	-2 13	12.7	ND	15.8	1.9	ND	2.7	8.5	ND	3.1	2.4	3.0	3.1	ND	1.6	ENTFC, STAE	IA
	28	-5 14	2.5	1.7	1.4	1.2	ND	1.5	1.0	1.0	0.9	1.4	2.2	1.8	ND	65.6	ENTFA, STAE	B

< 0.5	< 1	< 1.6	≥ 1.6	> 5	> 10	> 50
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titers against all bacterial species were observed. The antibody titers showed a broad inter-individual variation but were generally higher for *S. aureus*. This could be explained by the ubiquitous nature of *S. aureus*, where humans are regularly exposed to this microorganism, often within the first hours of life (Maayan-Metzger *et al.*, 2017; Verkaik *et al.*, 2010). Therefore, *S. aureus*-specific IgGs are regularly observed in healthy adults, independent of their carrier status, albeit at highly varying concentrations (Wertheim *et al.*, 2005). This was also reflected in the study healthy group, which comprised 6 *S. aureus* non-carriers, a persistent carrier and an intermittent carrier.

Sepsis is often caused by commensals or pathobionts, which belong to the physiological microbiota but can cause severe infection in immunocompromised hosts. Healthy humans gradually acquire systemic immune memory of those bacteria, which is reflected by the presence of specific IgG antibodies (Bröker *et al.*, 2014). These are probably induced by repeated minor bacterial invasions (Macpherson *et al.*, 2005), which explains the presence of specific antibodies in healthy adults, as well as the basal antibody levels among the patients included in the study. For instance, plasma antibodies could also be detected against the skin commensal *S. epidermidis*. The observation that *S. epidermidis* colonises humans more often than *S. aureus* but is less virulent and invasive (Massey *et al.*, 2006) is consistent with the present study data (Fig. 1).

The anti-bacterial antibodies in healthy subjects were highly variable in terms of binding patterns and titers but changed little over time (Fig. 2 and Verkaik *et al.*, 2009). On the other hand, the present study showed that in most sepsis patients, the specific IgG levels increased considerably during the course of the infection. Similar observations have been reported in patients with *S. aureus* bloodstream infection, where these increases in IgG titers were clearly driven by the invasive pathogen (Kolata *et al.*, 2011). Many sepsis patients experienced a slight increase in anti-TT IgG binding (Fig. 2). However, this was – except for one case (patient 28) – much lower than that of the anti-bacterial response, confirming the generation of a species-specific IgG response. When sepsis patients were tested for changes in IgG binding to 9 common sepsis pathogens, the response patterns were highly individual and discriminated between different infectious agents. In 4 patients, the increase in IgG was selective for 1 single microbiologically-diagnosed pathogen. Interestingly, a case report has previously provided evidence that the anti-bacterial IgG response is even strain-specific. In an *S. aureus* carrier, the increase in antibody binding is specific for the extracellular proteins of the invasive clinical *S. aureus* isolate, rather than the colonising strain (Kolata *et al.*, 2011). Although cross-reactions between related bacterial species are expected, especially when working with complex bacterial protein extracts, the comparison of increases in antibody titers against the

three tested staphylococcal species often showed a dominant reaction against only one of them (Table 3). The same holds true for Gram-negative bacteria, where no obvious cross-reactivity among the different species was observed but rather, a species-specific immune response.

In the present proof-of-concept study, 20 out of 37 sepsis patients with a microbiological diagnosis developed a humoral immune response against the pathogen diagnosed by either microbial culture or PCR. Remarkably, in 15 of these patients there was an antibody response to additional undiagnosed bacteria. Consistent with the serological findings, most of those patients suffered from an intra-abdominal infection due to gut perforation or surgical suture failure. Hence, the immunological approach was often more sensitive than conventional microbial culture-based diagnostics, indicating a poly-microbial infection. In patient 41, for instance, *E. faecium* was microbiologically identified; however, the increase in IgG binding to *S. marcescens* exceeded that observed for the diagnosed pathogen by 10-fold. This could have two explanations: either *S. marcescens* was the actual cause of infection or the basal anti-*S. marcescens* antibody levels were very low when compared to those against *E. faecium*. Since the baseline situation significantly influenced the magnitude of the calculated ratio, this could lead to an overestimation of the observed antibody increase for *S. marcescens*. It is worth mentioning that *S. marcescens* was diagnosed in the blood culture of a single patient (patient 36), but not by PCR. An immune reaction against pathogens undetected by microbiological diagnosis could explain cases of failed antimicrobial therapy despite a seemingly targeted selection of antibiotics.

The study further demonstrated that, while the pre-existing anti-bacterial antibody titers were highly variable (Fig. 1), increases in anti-bacterial IgG were indicative of an infection and specific to the infectious agent. Similar observations were made in children with musculoskeletal *S. aureus* infections. Although this young cohort presented with low basal anti-*S. aureus* antibody levels, the diagnostic power of antibody titer changes was higher than that of single measurements at diagnosis (Wood *et al.*, 2018).

The present proof-of-concept study has some limitations. First, the healthy subjects were not strictly sex- and age-matched to the sepsis patients. Second, 17 out of 37 patients showed no increase in specific plasma IgG against the microbiologically diagnosed pathogen, which might be for various reasons:

- the microbiologically diagnosed pathogen did not cause the infection, but was merely a contaminant and, hence, did not elicit an immune response;
- at the first time point of antibody determination, the disease process was already too advanced to observe an appreciable increase;
- serum conversion occurred after the second blood sample was collected;
- the patients were unable to generate an efficient immune response due to immunosuppression;

- the immediate initiation of an appropriate antibody therapy reduced the bacterial load so rapidly that no immune response was triggered. Nevertheless, the study clearly showed that a focus on the immunological host response to sepsis-causing agents can add valuable information to microbiological and molecular diagnostics, which target the pathogen. It offers several advantages over those established techniques that might warrant the implementation of this approach alongside routine diagnostics. For instance, it is often challenging to cultivate pathogens from biofilms of PJI, where culture-based methods would require invasive surgery to access the pathogens. In such cases, a serological non-invasive approach would be of great benefit. Moreover, its readout is robust against antibiotic treatment and it can discriminate immunologically relevant infection from sample contamination.

In the future, efforts will be made to improve and optimise both the sensitivity and the selectivity of this approach, by supplementing the complex antigen mixtures with panels of recombinant species-specific antigens, in order to clearly distinguish between immune reactions against closely related species, such as *S. aureus* and *S. epidermidis*.

### Conclusion

Increases in antibacterial antibodies provided valuable insights into the infection process. While in an acute infection the antibody response develops too late for early pathogen identification and guidance of the initial antibiotic treatment, it can help validate the diagnosis, especially in patients with chronic infections that often occur in association with indwelling medical devices/materials. The extent to which higher antibody titers against pathobionts provide clinical protection and could inform the development of immunisation strategies against pathobionts remains to be investigated.

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The authors have no conflicts of interest to declare.

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### Discussion with Reviewer

**Gowrishankar Muthukrishnan:** What is the rationale for choosing an immunoblot assay as opposed to other approaches to assess IgG responses?

**Authors:** The Peggy Sue™ Simple Western Assay is a semi-automated quantitative immunoblotting that allows for the simultaneous analysis of up to 88 samples in a short time. Moreover, only small sample volumes are required, which is especially important

when working with patient material. The results can be displayed as electropherograms, indicating the antibody binding profiles with information about the MW of the sample, or can be automatically transformed into area under the curve (AUC) for quantification. Therefore, the Peggy Sue™ Simple Western Assay is superior to an enzyme-linked immunosorbent assay (ELISA). Nevertheless, the assay has some limitations. Only one analyte can be measured at a time. In the future, we hope to improve the assay by using recombinant proteins of different pathogens and by using the Luminex technology to quantify the antibody response against several antigens simultaneously.

**Editor's note:** The Scientific Editor responsible for this paper was Fintan Moriarty.