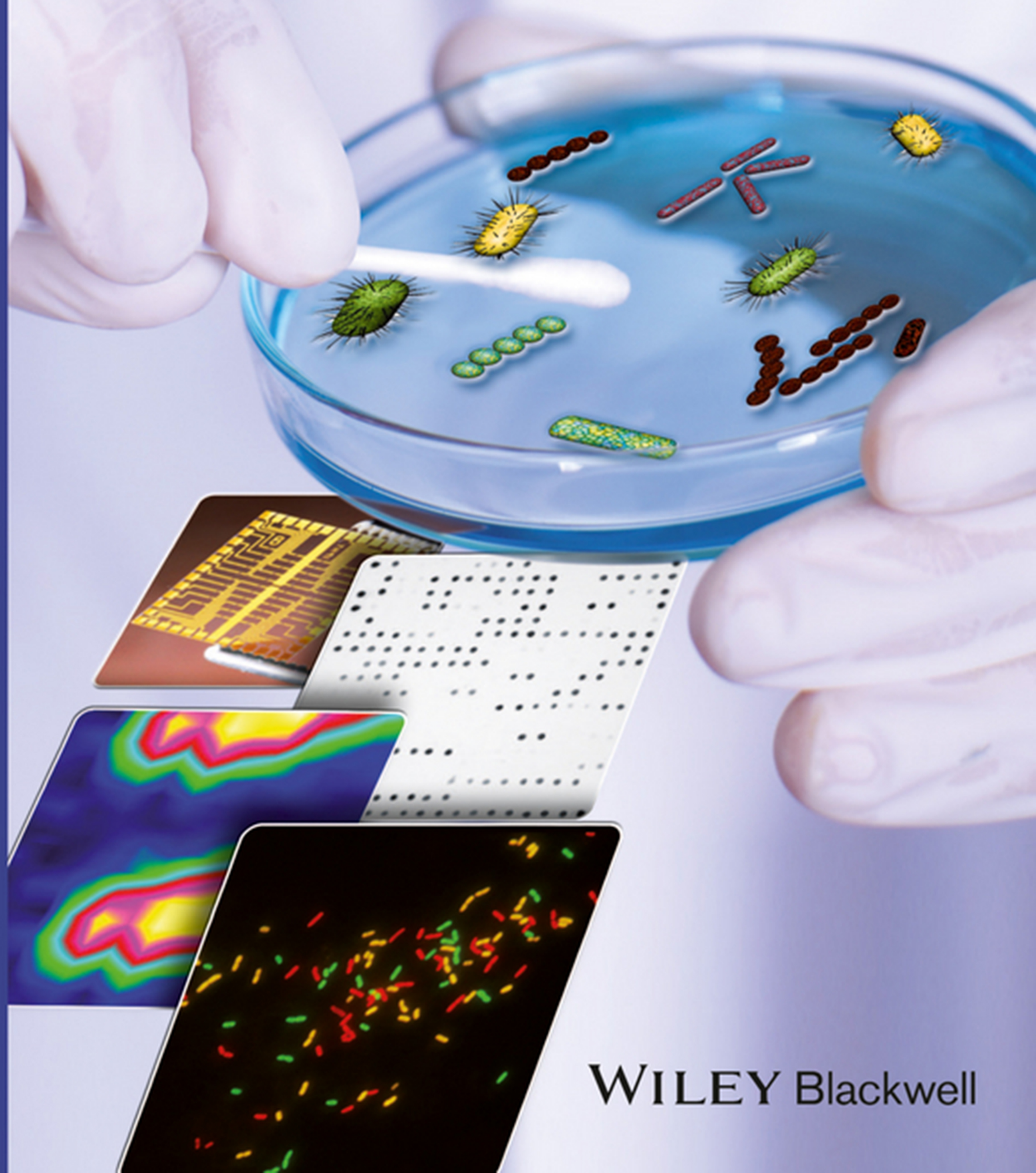
Modern Techniques for Pathogen Detection

Edited by Jürgen Popp
and Michael Bauer



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Preface

This book gives a comprehensive overview of the frontline research in pathogen detection. Infectious diseases caused by bacterial, viral, or fungal pathogens are one of the leading causes of death worldwide. In order to find the optimal treatment regime for the highly heterogeneous group of patients and to significantly reduce costs in medical care, novel tools that characterize the pathogenic microbes and the host-specific immune response are highly desired. Information about the phenotype and genotype of the germs help to accelerate quick and effective interventions, for example, the treatment of a sepsis patient with the right medication. Especially, within this context, the terms personalized medicine and pharmacogenomics, which define a stratified treatment for each individual patient, are of increasing importance. Due to a high variety in the responses of a patient panel to similar drug treatment regimes, which are caused by genetic and/or physiological variations among patients, the therapy has to be adapted to achieve highest efficiency and lowest side effects.

A plethora of analytical methods such as spanning microbiology, biochemistry, molecular biology, immunology, and biophysics are commonly applied for the detection of pathogens. *Modern Techniques for Pathogen Detection* informs in detail about various cutting-edge tools for pathogen identification. International scientists of the respective areas highlight recent emerging methods as well as improvements of conventional assays within individual chapters.

The management of life-threatening infections, especially sepsis, is covered, due to its importance, in a separate chapter of the book (Chapter 1). In the case of a sepsis suspicion, pathogen detection and administration of the appropriate drug combination should be performed as soon as possible. We stand at the beginning of a more personalized approach toward sepsis care. The novel developed analytical tools can facilitate an early and precise diagnosis of the pathogenic causatives of the severe infection concomitant, with profiling of their genetic status in terms of resistance to a certain antimicrobial therapy and finally also the monitoring of the treatment response.

Conventional techniques, involving microscopic or culture-based identification, enable the reliable detection of bacteria at low concentrations (Chapter 2). The morphological rating of bacteria from human patient samples combining microscopy with several classical or immunological staining techniques is an

inherent part of everyday clinical practice. Beyond that, the gold standard in routine pathogen diagnostics is based on culture methods, which use selective growth media in combination with a multitude of biochemical tests. These tests are inexpensive and provide qualitative and quantitative information about the investigated pathogens. Culture-based techniques usually require an overnight incubation, thus the analysis time is extended to at least 1 day. In addition, the culture-based approach relies on the presence of culturable cells. Therefore, only the implementation of testing results from other assays provides the possibility to evaluate the pathogenic flora in case of non-culturable or slow-growing organisms.

In the last decades, molecular assays improved pathogen detection, leading to higher sensitivity, selectivity, and a significantly reduced sample-to-answer time. Nucleic acid-based techniques are discussed in detail in the relevant chapters (Chapters 3, 4, and 7). Great efforts were made to enable a simultaneous screening of several pathogens by means of multiplex nucleic acid amplification or microarray platforms. The trend to integrate automation in the molecular assay workflow has led to sample-to-answer times of several hours, depending on the employed diagnostic technique. Nevertheless, the results of PCR and microarray approaches should be critically rated in the clinical and microbiological context of the patients. An elegant assay is the direct specification of pathogens within a blood culture by means of fluorescence *in situ* hybridization (FISH). This approach is a follow-up staining method after the initial identification of Gram-positive or Gram-negative bacteria. A Quick-FISH testing platform can minimize the sample-to-answer time starting from Gram stain to 30 min. Of great importance is the fact that the inclusion of nucleic acid-based techniques into clinical routine depends on both the total hands-on-time and operator-friendly analysis platforms.

Furthermore, modern analytical methods, such as MALDI-TOF mass spectrometry, are discussed as a routine clinical microbiology laboratory for identification of infectious agents (Chapter 5). This fast evolving and increasingly applied technique allows the rapid and accurate identification of microorganism as well as antibiotic resistance markers based on specific biomarker signatures. Within this context, this pattern-matching process offers the balance between the time-consuming culture-based techniques and highly accurate nucleic acid-based techniques. Very promising tools for pathogen profiling are IR and Raman spectroscopic techniques, which are therefore also introduced within the book (Chapter 6). Their main advantage is given by the possibility of probing bacteria at single-cell level without any prior cultivation steps. IR and Raman spectroscopic techniques are successfully applied to identify bacteria by matching experimental molecular fingerprint information of single bacteria with reference members of the same biological species in preformed spectral databases by chemometrics. This pattern-matching approach is described for the direct classification and identification of infectious agents.

In summary, *Modern Techniques for Pathogen Detection* highlights state-of-the-art knowledge, emerging trends, and critical advices from experts for pathogen

detection. We highly recommend the book to interdisciplinary working scientists at the nexus of fundamental research and clinical application, which plan to focus their research on further improvements that enable accurate and timely pathogen identification along with the respective antibiotic resistance information. The latter one is definitely the prerequisite to quickly adjust the right medication for a patient who suffers from severe microbial infection. A further crucial challenge for all the applied tools in terms of pathogen detection is the precise distinction of normal colonization and pathogenic invasion.

Last but not least, we would like to thank all the authors for the enjoyable cooperation that contribute to this book. The authors have a designated longtime expertise on their respective scientific area within the fast evolving field of microbial infection detection. Special thanks for their conscientious work, the abundance of patience, and the countless fruitful discussions. We would also thank the dedicated team of the publisher.

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1
Unmet Medical Needs in Life-Threatening Infections – Caring for the Critically Ill

Michael Bauer, Andreas Kortgen, and Mervyn Singer

1.1
Life Threatening Infections and Sepsis – Defining the Problem

The large number of infectious agents, complicated further by many varied pathogen- and host-specific characteristics, results in a broad spectrum of communicable diseases of which both prevention and control are challenging. While many infectious diseases are benign and are primarily treated in the community, severe infections may give rise to an urgent need to control the source of infection, to implement appropriate anti-infective therapy, and to provide supportive care to maintain homeostasis [1].

Under these conditions, the patient outcome from infection is determined not only by the invading pathogen which can be directly toxic and destructive to cells and tissues but also – or even primarily – by the host response. This host response may be inappropriately exaggerated, leading to severe tissue injury. Here, the effector molecules of immune cells, such as oxygen free-radicals and nitric oxide, cannot discriminate between microbial targets and host tissue [2]. Indeed, a novel concept has been proposed to describe the development of organ failure, that is, severe sepsis, as a disturbed "disease tolerance" where the eventual development of organ dysfunction is considered an inability to establish an appropriate equilibrium between direct pathogen damage and the ensuing host response (Figure 1.1) [3]. Patients with an uncontrolled focus of infection or an exuberant host response are particularly prone to develop organ dysfunction requiring care in a specialized "intensive care unit (ICU)." Such patients are referred to as *septic* (Figure 1.2).

Sepsis is defined and diagnosed by nonspecific alterations in temperature, heart and respiratory rate, and white cell count secondary to infection (Table 1.1) [4]. Unfortunately, in current clinical practice, neither the causative pathogen nor the specific cellular processes underlying deterioration of organ function that would be amenable to specific therapeutic intervention can be assessed in a way that would allow tailoring of anti-infective or immunomodulatory therapies to specific patient needs. This is particularly relevant given the pressing need to respond within the first few "golden" hours. These shortcomings regarding "point-of-care" diagnostics are in sharp contrast to the burgeoning development of sophisticated

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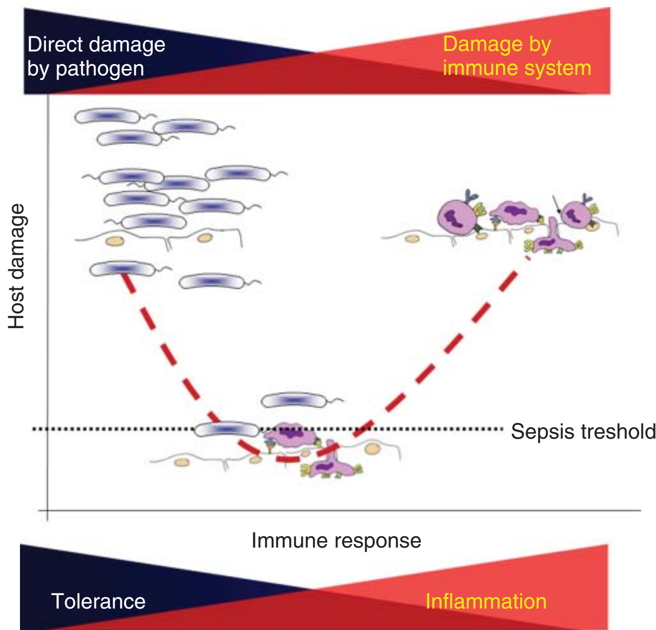

Figure 1.1 Evolving concepts of sepsis as a "host defense failure disease." The host response to invading pathogens requires a cytotoxic response that can result in a trade-off where tolerance of a pathogen may be associated with less organ injury.

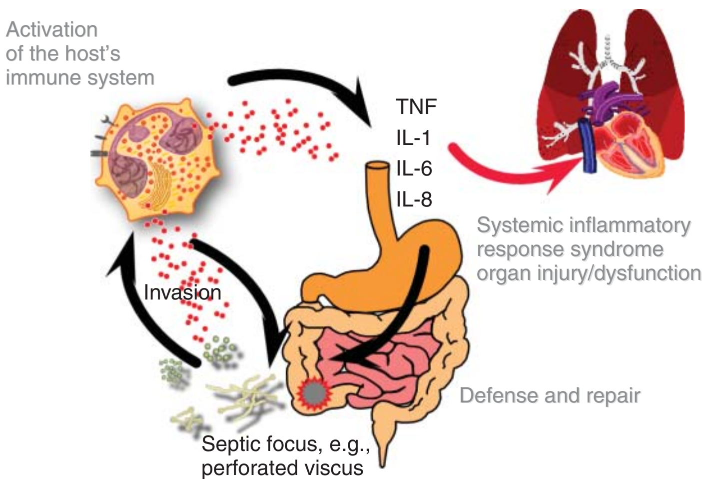

Figure 1.2 Activation of the innate immune system as a "double-edged sword." Activation of innate immunity reflects a prerequisite for defense and repair of a septic focus, such as a perforated viscus. However, this may lead to collateral damage if spillover of inflammatory mediators or release of activated cells into the systemic circulation occurs.

Table 1.1 Diagnostic criteria for the "systemic inflammatory response syndrome" (SIRS criteria).

- Temperature >38 or <36 °C
- Heart rate >90 beats/min
- Respiratory rate >20 beats/min or p_aCO_2 <32 Torr (4.3 kPa)
- White blood count >12 000 cells/mm^3 or <4000 cells/mm^3 or >10% immature (band) forms

molecular tools and the improved molecular and cellular understanding of the pathogen–host interaction via specific receptors and signaling cascades [5, 6]. As stated by Nathan [2]: "it makes no sense to use twenty-first century technology to develop drugs targeted at specific infections whose diagnosis is delayed by nineteenth-century methods." Thus, development of innovative diagnostic tests and strategies are needed to optimize treatment strategies, not only in selecting the correct anti-infective agent but also modulating inflammatory and other responses to fundamentally improve outcomes in a "personalized" manner.

The resulting diagnostic uncertainty regarding the causative pathogen reflects a central dilemma of intensive care physicians in treating life-threatening infections. On one hand, there is an important need to avoid delays in the initiation of appropriate antibiotics [7], yet this, in turn, triggers the overuse of "broad spectrum" antimicrobial agents creating a tremendous problem with multiresistant pathogens [8]. Likewise, many septic patients may already be in a state of overall immune suppression at the point of admission to intensive care, as anti-inflammatory systems are also activated in sepsis and these may outweigh the proinflammatory response. Introduction of an anti-inflammatory agent to such patients may arguably compromise the host even more.

1.2 Sepsis as a "Hidden Healthcare Disaster"

Sepsis arises from community-acquired infections but also, and more frequently, from healthcare-associated infections. It is a leading cause of morbidity and mortality worldwide. Its incidence is increasing, and the overall mortality is now in a similar range to that of myocardial infarction or stroke [9, 10]. This likely reflects changing demographics, with an aging population. In parallel, an ever-increasing number of invasive procedures, including those directly affecting the immune system, such as antineoplastic chemotherapy or organ transplantation, are performed in patients who would previously not have been considered for such procedures. As a consequence, the rate of hospitalization for sepsis in the United States increased from 221 per 100 000 population in 2001 to 377 per 100 000 in 2008 (Figure 1.3) [9]. A similar increase in the incidence of severe postoperative sepsis is also noted [11].

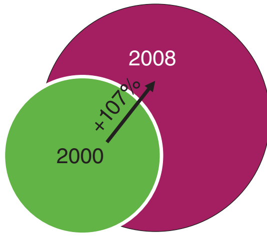

Figure 1.3 Sepsis – an underestimated and silently growing problem of modern healthcare: Because of multiple factors related to demographic changes, an increasing invasiveness of procedures in patients with inherent impaired immune function, and the advent of multidrug resistance in particular to Gram-negative pathogens, there is a silent but dramatic increase in the incidence of sepsis in health-care systems across the world.

Sepsis has been called a "hidden public health disaster" [12]. Survivors carry an under-recognized risk of long-term cognitive and physical disability [13] and a more than twofold risk of dying over the next 5 years compared with appropriate controls [14]. The Center of Disease Control recently estimated that 15 billion dollars were spent on hospitalizations for sepsis alone in the United States and that inflation-adjusted aggregate costs for treating such patients increased annually by more than 10% [9].

1.3
Microorganisms and Types of Infection Triggering Sepsis

A recent global picture of infection and sepsis in ICUs worldwide is provided by the "Extended Prevalence of Infection in Intensive Care" (EPIC II) study. This reflects a 1-day, prospective, point prevalence study conducted on May 8, 2007, with subsequent follow-up [15]. Demographic, physiologic, bacteriologic, therapeutic, and outcome data were collected from approximately 14 000 patients in 1265 participating ICUs from 75 countries. These included 667 Western European ICUs, 210 Central and South American, 137 Asian, 97 Eastern European, 83 North American, 54 Oceanic, and 17 African. Sixty percent of participating ICUs were situated in university hospitals, 66% were mixed medical-surgical ICUs, and 94% had 24 h ICU physician coverage. On the study day, approximately half the patients were considered infected and 71% were receiving antibiotics. Infection was mostly of respiratory origin (66%), followed by abdomen (20%), bloodstream (15%), and renal tract/genitourinary system (14%). Microbiological cultures were positive in 70% of the patients with presumed infection, with 62% of positive isolates being Gram-negative organisms, 47% Gram-positive, and 19% fungi. The most common Gram-positive organism isolated was *Staphylococcus aureus* (20%), while the commonest Gram-negative organisms were *Pseudomonas* species (20%) and *Escherichia coli* (16%). Patients who had been in ICU for longer prior to the study day had higher rates of infection, especially with resistant and thus more difficult-to-treat pathogens, such as *Staphylococci*, *Acinetobacter*, *Pseudomonas*, and *Candida* species. Of note, ICU mortality (25% vs 11%) and hospital mortality (30% vs 15%) of infected patients was more than twice that of noninfected patients. Other, albeit smaller, surveys corroborate the EPIC II data and confirm the disease burden of infection in the critical care setting which increases with

the duration of stay as well as with shifting patterns of microorganisms [16]. Since 2007, a substantial increase in difficult-to-treat infections has been observed. This is primarily attributable to multidrug-resistant Gram-negative bacteria, a proportion of which are virtually untreatable, such as some carbapenemase-producing *Klebsiella* strains [17].

1.4 Emerging Problems Related to Resistance in Bacterial Infections

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains an important cause of healthcare-associated infections, and is endemic in most hospitals. Healthcare-associated MRSA infections are associated with increased morbidity and mortality compared to infections caused by methicillin-susceptible strains [18, 19]. MRSA is also an increasingly important cause of infection in the community setting. MRSA infections, both healthcare- and community-associated, are generally caused by a very limited number of (clonal) strains, suggesting that most cases result from direct or indirect person-to-person transmission of MRSA [20, 21]. The major reservoir for transmission is likely to be infected or colonized patients, with the vector being healthcare personnel or contaminated, shared equipment. With the introduction of a variety of bundled strategies including, but not restricted to, careful hand hygiene, there has been an associated reduction in the burden of MRSA infection in the healthcare setting. In 2005, there were an estimated 94 000 MRSA infections in the United States associated with nearly 18 000 deaths. Of these, 86% were associated with healthcare delivery, two-thirds of which had their onset outside the hospital setting [22].

Despite measures that have successfully prevented and controlled healthcare-associated MRSA, the number of Gram-negative bacterial infections continues to grow [23]. This is compounded by an increasing problem of antibiotic resistance of these pathogens which may result in higher mortality and morbidity [24].

There are thus conflicting recommendations. On one hand, there is advice to administer appropriate and early empirical antibiotic therapy, especially in patients with risk factors such as compromised immune function. In view of the growing resistance problem, multiple broad-spectrum antibiotics are often proposed. On the other hand, there are strong recommendations to limit antibiotic usage in general. This is a clear testimony for the need of diagnostic tests with fundamentally improved performance regarding sensitivity, specificity, and, most importantly, time-to-result.

1.5 The Role of Fungi and Viruses

Bacteria dominate as the type of pathogen responsible for most life-threatening infections in the "immune competent" host. However, immunosuppression

occurring in the later phases of sepsis [25], along with shifts in the host's commensal flora (primarily induced by antibiotics), contributes to overgrowth by fungi, most notably *Candida* species, in sterile body compartments. This can give rise to difficult-to-diagnose infections which may also contribute to the overall death toll. In a recent retrospective chart review study, we identified 999 patients with severe sepsis or septic shock from a total of 16 041 patients admitted to our 50-bed surgical ICU in a single center; hospital mortality was approximately 30% [16]. In total, data from 2117 blood cultures were available for analysis. Three phases could be described based on peaks in mortality. A third of all deaths occurred in the first 5 days following ICU admission. Of 882 blood cultures drawn within the first 5 days, only 15% were positive. Of note, 524 blood cultures were drawn in those patients staying >2 weeks and, while positive blood cultures were less frequently observed, the rate of opportunistic bacteria and *Candida* species doubled from 9% in the acute phase to 18% in this later phase.

While the role of invasive fungal infection is increasingly acknowledged, the contribution of viral infections to initiate or maintain a systemic inflammatory response syndrome SIRS is poorly defined. The 2009 influenza A (H1N1) pandemic did not significantly affect ICU occupancy rates and, compared with community-acquired pneumonia of other origins, H1N1 pneumonia was associated with the same risk of death when potential confounders were taken into consideration [26]. However, this pandemic more commonly affected young people, many of whom developed severe respiratory failure requiring extracorporeal lung assist support. As the pathogen underlying ICU admission for community-acquired pneumonia is rarely identified with conventional diagnostics, viral infections are probably underdiagnosed. Viruses may play an important role in complicating the course of defined ICU patient populations, such as cytomegalovirus (CMV) in immunocompromised patients. While reactivation of dormant virus within the critically ill host likely occurs, it remains unclear to what extent they cause secondary infections. Antiviral treatment may improve outcome [27], but they do carry their own toxicity. Multicenter trials that address this problem are ongoing. Ganciclovir is frequently used as both first-line prophylaxis and systemic disease therapy against CMV, but resistance is increasingly occurring and this is associated with worse outcomes. Thus, implementation of rapid and sensitive techniques for the early detection and monitoring of CMV and ganciclovir resistance is clearly desirable to support patient management [28]. Furthermore, strategies to individualize the therapy of life-threatening infection by viruses such as CMV and Epstein–Barr virus may include, in addition to antiviral agents, either a reduction in immunosuppressant therapy (as these infections occur frequently in post-transplant patients [29]), or even immunoactivating agents such as Granulocyte macrophage colony-stimulating factor GM-CSF and interferon-gamma. Here, a reliable point-of-care monitoring of the host's immune system would potentially allow a correct selection of agents to improve clearance of infection while at the same time reducing the risk of, for example, graft failure in transplant patients [30].

1.6 The Need for New Approaches in Diagnostics of Life-Threatening Infection and Sepsis

The presence of an infectious focus is currently identified and confirmed by a combination of clinical examination and imaging techniques. Whenever possible, specimens are obtained from the site of infection in addition to blood cultures for conventional microbiology. An example is taking specimens during lung washings during bronchoscopy for suspected lower respiratory tract infection. At present, these techniques, though time consuming, allow a better determination of the infecting pathogen and antibiotic resistance patterns [31].

The polymerase-chain reaction (PCR) technique can directly amplify pathogen DNA from a suspected focus [32, 33] or a blood sample. This carries the potential to increase the sensitivity of pathogen detection and to decrease the result turnaround time in routine clinical practice [34]. At present, PCR testing is generally considered as supplementary to culture-based techniques, particularly for fastidious, resistant, and difficult-to-culture pathogens. Some experts suggest that the focus for such tests should be on detection of pathogens or resistance factors that fall outside guideline-recommended antibiotic coverage, as well as for specific at-risk populations, for example, transplant recipients [35]. However, with improvements in technology, point-of-care PCR testing of blood and other body samples may not only shorten the time to diagnosis but also reduce the number of patients receiving inappropriate empirical antibiotics.

Although inappropriate anti-infective therapy is seemingly associated with excess mortality [36, 37], a liberal or "aggressive" strategy with early initiation of (combined) antibiotics to cover a very broad spectrum of pathogens may also be associated with similar increases in mortality [38]. Although not fully understood, there is increasing support for the concept that concomitant release of host intracellular "danger-associated molecular patterns (DAMPs)" or "alarmins" [39] can signal, via the Toll-like and other receptor systems, a similar pathophysiological cascade culminating in multiple organ failure as that induced by "pathogen-associated molecular patterns (PAMPs)" released from bacteria and other pathogens [40, 41]. Indeed, DAMPs may be released even in the absence of pathogens or their PAMPs. The use of anti-infective agents themselves carries multiple adverse effects (Table 1.2); this is further compounded by altered handling of these drugs by the dysfunctional liver and/or kidney, which not only

Table 1.2 Problems associated with antibiotic use in individual patients.

- Overgrowth of (multi)-drug resistant bacteria and fungi
- Jarisch–Herxheimer reaction: release of bacterial products, such as endotoxin potentially triggering a vigorous host response with associated side effects on organ function
- Effects on critical cellular effector functions: immunomodulatory effects, impairment of mitochondrial function
- Typical drug-related side effects: for example, rashes, liver, and renal dysfunction

makes pharmacokinetics and dosing unpredictable but also increases the risk of direct drug-induced toxicity [42].

1.7
Rapid and Sensitive Culture-Independent Strategies to Identify Blood Stream Infection

The basic principles regarding the diagnostics of infection hold particularly true for blood cultures, which is the current gold standard for identifying primary or secondary bloodstream infection. However, this is far from being an ideal gold standard, as a positive result is obtained in only a subset of severely septic patients and results are frequently obtained too late to influence clinical decision making [43, 44].

Molecular approaches to improve conventional culture-based identification may range from strategies to shorten the time from positivity of the blood culture to identification of the pathogen to complete culture-independent, direct microbial nucleic acid amplification techniques.

Important developments to improve the performance of the blood culture approach include fully automated instruments for handling and culture and the use of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, which can decrease the time-to-result to approximately 1 h after a positive culture is recognized [44].

An alternative strategy, that is, extraction and amplification of microbial nucleic acids from a positive blood culture and subsequent hybridization on a microarray platform to detect the pathogen and certain resistance genes (gyrB, parE, and mecA) among 50 bacterial species (Prove-it Sepsis, Mobidiag, Helsinki, Finland), was recently evaluated [45]. A total of 2107 positive blood culture samples taken from 3318 blood samples from patients with suspected sepsis were analyzed; 86% of positive blood culture samples included a pathogen covered by the molecular assay which had an overall 94.7% sensitivity and 98.8%, specificity; both increased to 100% for identifying MRSA bacteremia. On average, the assay was 18 h faster than conventional blood cultures, providing proof of the concept that molecular assays can shorten the time-to-result. Shortcomings included an incomplete coverage of pathogens, an inability of the test to be applied directly to a biological sample, and restricted information regarding antimicrobial susceptibility (primarily regarding multiresistant Gram-negative bacteria) despite an excellent performance for detecting MRSA. While PCR-based detection of MRSA (and also Vancomycin-resistant Enterococci VRE) is feasible because of a limited number of resistance genes, the need to identify the large and continuously evolving set of genotypes encoding extended-spectrum β-lactamases renders a molecular approach difficult and a conventional PCR-based approach unreliable.

These shortcomings, obviously with the exception of the need for prior culture, also hold true for PCR-based approaches to directly amplify microbial nucleic acids from the bloodstream. This led to the view discussed earlier that PCR tests should only supplement but not replace blood culture (BC). However, such a