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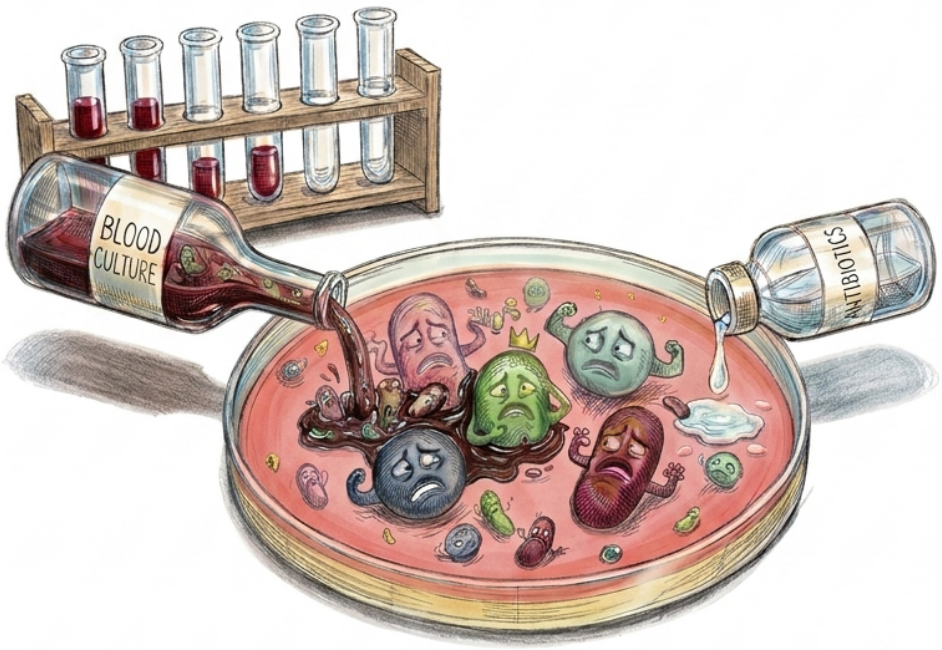
Improving microbiological diagnostics in bacteraemia

Rapid antimicrobial susceptibility testing, diagnostic workflows and clinical impact

Anna Åkerlund



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Improving microbiological diagnostics in bacteraemia

Rapid antimicrobial susceptibility testing, diagnostic workflows and clinical impact

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Linköping 2026

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Abstract

Early recognition and management of severe infections, including timely administration of appropriate antimicrobial therapy, are critical determinants of patient outcome. Timely microbiological diagnostics are essential for the management of patients with bloodstream infections. While blood culture remains the cornerstone of bloodstream infection diagnosis, culture-based methods are often associated with long turnaround times (TATs) that may delay adequate antimicrobial therapy. Rapid antimicrobial susceptibility testing (RAST) methods and optimised diagnostic workflows have the potential to shorten the time to actionable microbiological results and facilitate earlier targeted treatment. This thesis aimed to evaluate approaches for accelerating antimicrobial susceptibility testing (AST) and optimising blood culture diagnostic pathways.

In **Study I**, we demonstrated that the newly developed EUCAST RAST method, designed for the most common pathogens causing bloodstream infections and performed directly from positive blood cultures, can be implemented in routine clinical microbiology laboratories (CMLs) without substantial training or additional investments. Across participating laboratories, the method achieved a categorical agreement of 97% compared with the reference method while providing antimicrobial susceptibility results within 4-6 hours after blood culture positivity.

In **Study II**, we demonstrated that early reading of disk diffusion inhibition zones following shortened incubation is feasible for *Haemophilus influenzae* and *Streptococcus pneumoniae*, with categorical agreement exceeding 95% compared with standard AST. By applying Areas of Technical Uncertainty (ATUs), the risk of misclassification was substantially reduced, supporting reliable susceptibility testing of common fastidious pathogens within a single working day.

In **Study III**, we identified considerable variation in blood culture diagnostic services among Nordic CMLs. Despite improvements in

rapid species identification compared with a Swedish survey performed five years earlier, several organisational and logistical barriers affecting timely blood culture diagnostics remained, indicating substantial potential for further optimisation.

In **Study IV**, optimisation of the blood culture diagnostic pathway shortened diagnostic TATs and facilitated earlier treatment optimisation, reducing the time to adequate antimicrobial therapy by up to 22 hours. Decentralised blood culture incubation enabling 24/7 loading and incubation of blood culture bottles was identified as the single most effective intervention for reducing diagnostic delays.

In conclusion, microbiological diagnostics in bacteraemia can be accelerated through both rapid species identification and AST methods, as well as optimisation of blood culture diagnostic workflows. Such measures shorten the time to actionable microbiological results and facilitate earlier adequate antimicrobial therapy, thereby improving the management of patients with bloodstream infections.

Keywords: Blood culture, bacteraemia, antimicrobial susceptibility testing, rapid diagnostics, diagnostic turnaround time, antimicrobial therapy, sepsis.

Populärvetenskaplig sammanfattning

Svåra infektioner och blodförgiftning (sepsis) kan utvecklas snabbt och vara livshotande. För att patienten ska få rätt behandling så tidigt som möjligt behöver läkaren veta vilken bakterie som orsakar infektionen och vilka antibiotika bakterien är känslig för. Trots stora framsteg inom laboratoriediagnostiken tar sådana analyser ofta lång tid eftersom bakterier först måste odlas fram innan de kan identifieras och resistensbestämmas.

Syftet med denna avhandling var att undersöka hur tiden från provtagning för blododling till färdigt mikrobiologiskt svar kan förkortas, både genom snabbare metoder för resistensbestämning och genom förbättringar av arbetsflöden inom blododlingsdiagnostiken.

I den första studien utvärderades en ny metod för snabb resistensbestämning direkt från positiva blododlingar. Resultaten visade att metoden kan användas på de flesta mikrobiologiska laboratorier utan omfattande utbildning eller investeringar och att tillförlitliga resistenssvar kan erhållas redan några timmar efter att blododlingen visat bakterieväxt.

I den andra studien undersöktes om resistensbestämning med en etablerad laboratoriemetod kan bedömas tidigare än idag för två bakteriearter (*Haemophilus influenzae* och *Streptococcus pneumoniae*) som är vanliga orsaker till luftvägsinfektioner och ibland även allvarliga invasiva infektioner. Studien visade att många resultat kunde bedömas och rapporteras en dag tidigare än med dagens standardmetoder, utan att säkerheten i tolkningen äventyrades.

Den tredje studien kartlade hur blododlingsdiagnostik bedrivs i de nordiska länderna. Resultaten visade stora skillnader mellan laboratorier när det gäller hur diagnostiken organiserades och vilka snabbdiagnostiska metoder som användes. Samtidigt identifierades flera områden där diagnostiken kunde effektiviseras ytterligare.

I den fjärde studien analyserades effekten av olika förbättringsåtgärder inom blododlingsdiagnostiken. Förändringarna

ledde till kortare svarstider och gjorde det möjligt att ge patienter mer träffsäker antibiotikabehandling tidigare. Den största effekten sågs när blododlingsflaskor kunde inkuberas (placeras i varma odlingsskåp) dygnet runt nära patienten, vilket minskade onödiga väntetider i diagnostikkedjan.

Sammanfattningsvis visar avhandlingen att tiden till viktiga mikrobiologiska svar kan förkortas genom både snabbare laboratoriemetoder och förbättrade diagnostiska processer. Snabbare diagnostik ger bättre möjligheter att tidigt välja rätt antibiotikabehandling och att identifiera orsaken till allvarliga infektioner, vilket kan bidra till förbättrad vård för patienter med blodförgiftning och andra svåra infektioner.

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Declaration of AI-Assisted Technologies

During the preparation of this work, the author used Microsoft Copilot, an AI-assisted tool, to support language editing, text revision, and limited literature exploration. All sources and factual information were independently verified against the original publications. After using the tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the thesis.

List of papers

I. **Åkerlund A**, Jonasson E, Matuschek E, Serrander L, Sundqvist M, Kahlmeter G; RAST Study Group. EUCAST rapid antimicrobial susceptibility testing (RAST) in blood cultures: validation in 55 European laboratories. J Antimicrob Chemother. 2020 Nov 1;75(11):3230-3238. doi: 10.1093/jac/dkaa333. PMID: 32789506; PMCID: PMC7566356.
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II. **Åkerlund A**, Serrander L, Sundqvist M. Short incubation of disc diffusion for *Streptococcus pneumoniae* and *Haemophilus influenzae* to reduce time to susceptibility report. J Antimicrob Chemother. 2023 Oct 3;78(10):2563-2571. doi: 10.1093/jac/dkad272. PMID: 37667593; PMCID: PMC10545500.
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III. **Åkerlund A**, Petropoulos A, Malmros K, Tängdén T, Giske CG. Blood culture diagnostics: a Nordic multicentre survey comparison of practices in clinical microbiology laboratories. Clin Microbiol Infect. 2022 May;28(5):731.e1-731.e7. doi: 10.1016/j.cmi.2021.09.003. Epub 2021 Sep 17. PMID: 34537364.
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IV. **Åkerlund A**, Fredriksson M, Sundqvist M, Serrander L. Impact of stepwise improvements in blood culture diagnostics on turnaround time and time to adequate therapy: a retrospective longitudinal single-centre study. *Submitted to The Journal of BMC Infectious Diseases.*

Related articles.

Åkerlund A, Sundqvist M, Hanberger H, Åhrén C, Serrander L, Giske CG. Svarstiderna kan kortas vid mikrobiologisk diagnostik av sepsis--Bättre öppettider på laboratorier och aktiv rådgivning ger snabbare terapi [Response times can be shortened in microbiological diagnosis of sepsis. Better opening hours in laboratories and active advice enables faster therapy]. Lakartidningen. 2015 Feb 10;112:C73S. Swedish. PMID: 25668800.

Ekwall-Larson A, Fröding I, Mert B, **Åkerlund A**, Özenci V. Analytical Performance and Potential Clinical Utility of EUCAST Rapid Antimicrobial Susceptibility Testing in Blood Cultures after Four Hours of Incubation. Microbiol Spectr. 2023 Apr 13;11(2):e0500122. doi: 10.1128/spectrum.05001-22. Epub 2023 Feb 21. PMID: 36809027; PMCID: PMC10100889.

Contributions

I. I participated in antimicrobial susceptibility testing, data analysis, interpretation of the results, and manuscript preparation.

II. I conceived and designed the study together with the co-authors, performed all laboratory experiments, analysed the data, and drafted the manuscript.

III. I co-developed the study concept and questionnaire, collected and managed the data, participated in data interpretation, and co-drafted the manuscript.

IV. I co-designed the study, collected and curated the data, performed the analyses, interpreted the results, and drafted the manuscript.

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Introduction

Sepsis and bacteraemia

Sepsis and septic shock are life-threatening complications of infection and major causes of morbidity and mortality worldwide. Despite advances in modern healthcare, both conditions remain associated with a substantial risk of death and place a considerable burden on healthcare systems.

Definitions

According to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Organ dysfunction is operationalised as an increase of ≥2 points in the Sequential Organ Failure Assessment (SOFA) score, which evaluates dysfunction across six organ systems: respiratory, cardiovascular, hepatic, coagulation, renal, and neurological (1, 2).

Septic shock represents a subset of sepsis associated with profound circulatory, cellular, and metabolic abnormalities and is defined by the requirement for vasopressor therapy to maintain a mean arterial pressure of ≥65 mmHg and a serum lactate level >2 mmol/L despite adequate fluid resuscitation (1, 3). In patients with suspected infection, an increase in SOFA score of ≥2 points are associated with an in-hospital mortality exceeding 10% while the mortality rate among patients with septic shock exceeds 40% (2, 3)

Bacteraemia is defined as the presence of viable bacteria in the bloodstream. It may occur as a consequence of localised or systemic infection and can range from transient, clinically insignificant episodes to severe bloodstream infections associated with sepsis and septic shock.

While bacteraemia refers to the microbiological presence of viable bacteria in the bloodstream, bloodstream infection describes the clinical syndrome associated with microbial invasion of blood.

Bacteraemia may occur in patients with localised infection, bloodstream infection, sepsis, or septic shock, but is neither required for nor synonymous with sepsis (4).

Epidemiology and burden of disease

Estimating the incidence of sepsis and septic shock is challenging, as reported rates vary considerably depending on study design, data sources, case ascertainment, and the sepsis definitions applied. According to the Global Burden of Disease Sepsis Collaborators, an estimated 49 million sepsis cases occurred worldwide in 2017, corresponding to an incidence of approximately 623 cases per 100,000 person-years. Sepsis accounted for an estimated 11 million deaths, representing 19.7% of all deaths globally. The highest incidence and mortality rates were observed in sub-Saharan Africa, Oceania, South Asia, East Asia, and Southeast Asia, with the greatest burden generally occurring in low-income settings, where incidence rates are several-fold higher than the global average (5).

Following the introduction of Sepsis-3, population-based studies from Sweden and other high-income countries have reported sepsis incidence rates ranging from approximately 500 to 800 cases per 100,000 person-years and an in-hospital mortality of 15-17%. Corresponding incidence estimates for septic shock range from approximately 10 to 80 cases per 100,000 person-years (6-8).

The impact of sepsis extends beyond mortality. Sepsis is associated with prolonged hospital stays, intensive care utilisation, and substantial healthcare costs. Although patients with severe sepsis and septic shock incur the highest per-episode costs, the greater number of patients with less severe disease contributes substantially to the overall burden on healthcare systems (5, 9).

Aetiology of sepsis and bloodstream infections

Sepsis may be caused by bacterial, fungal, viral, or parasitic infections. The relative contribution of different pathogens varies according to age, geographical region, healthcare setting, and source of infection

(1, 5). Globally, diarrhoeal diseases are the most common underlying cause of sepsis, whereas lower respiratory tract infections account for the highest sepsis-related mortality (5). In Swedish and other high-income country cohorts, respiratory tract infections are the most common infection focus, followed by urinary tract, intra-abdominal, and skin and soft tissue infections (6, 7, 10, 11).

Despite extensive microbiological investigations, an etiological pathogen remains unidentified in a substantial proportion of patients with sepsis. Among cases with an established microbiological diagnosis, bacterial pathogens predominate. Blood cultures remain the most important microbiological investigation in sepsis and bloodstream infections, although they are positive in only approximately 15-25% of patients with sepsis in contemporary cohorts (6, 7, 11). The relatively low proportion of positive blood cultures may be explained by factors including absence of bacteraemia despite clinical sepsis, previous antimicrobial therapy, low-grade or intermittent bloodstream invasion, suboptimal blood culture sampling, and infections caused by fastidious, non-cultivable, or non-bacterial pathogens (1, 4, 12)

Blood culture positivity is generally higher in urinary tract infections, hepatobiliary infections, and skin and soft tissue infections than in lower respiratory tract infections (13-16). Among patients with bloodstream infections and sepsis-associated bacteraemia, *Escherichia coli* and *Staphylococcus aureus* are consistently among the most frequently isolated pathogens, followed by *Streptococcus pneumoniae*, other Enterobacterales, enterococci and other streptococci (4, 6, 7, 10, 11, 17). The microbiological spectrum differs between community-acquired and hospital-acquired sepsis, with a greater contribution of healthcare-associated Gram-negative bacteria, enterococci, and multidrug-resistant pathogens in the latter setting (17, 18).

Antimicrobial therapy

Early administration of appropriate antimicrobial therapy is essential in the management of severe infections, including sepsis. Multiple

observational studies have demonstrated an association between delays in antimicrobial treatment and increased mortality, particularly among critically ill patients and those presenting with septic shock (19-22). Consequently, current international guidelines recommend immediate initiation of antimicrobial therapy in patients with septic shock or a high likelihood of sepsis-associated organ dysfunction (22).

More recent evidence suggests that the impact of treatment delay on mortality varies according to disease severity. While prompt antimicrobial therapy remains important, the survival benefit associated with antimicrobial administration within the first hour appears greatest in patients with septic shock and other forms of critical illness (20, 21, 23). In patients with suspected sepsis who do not present with shock, a limited period of continued diagnostic evaluation may be justified to confirm the diagnosis, identify the source of infection, and optimise antimicrobial selection without adversely affecting clinical outcomes (21-23)

Empirical versus targeted antimicrobial therapy

As microbiological results are rarely available when treatment decisions are made, antimicrobial therapy in patients with suspected sepsis is typically initiated empirically. The choice of empirical therapy is guided by the suspected source of infection, the expected microbiological aetiology, local resistance epidemiology, patient-related risk factors, and disease severity. However, empirical treatment may be inadequate if the presumed diagnosis or source of infection is incorrect, if the causative pathogen is resistant to the selected antimicrobial agent, or if the actual microbiological aetiology differs from that initially anticipated, including unusual pathogens or polymicrobial infections. Such situations are not uncommon, and reported rates vary considerably across studies and clinical settings owing to differences in patient populations, infectious syndromes, causative pathogens, and definitions (24, 25).

Observational studies of bloodstream infections have demonstrated an association between non-covering empirical therapy and increased mortality (25, 26). Similarly, inadequate empirical antimicrobial therapy has repeatedly been associated with adverse outcomes, including increased mortality, prolonged hospital stay, and higher healthcare costs in patients with severe infections and sepsis (24, 27, 28). As a result, empirical antimicrobial therapy is often initiated with broad-spectrum agents. However, empirical therapy may be unnecessarily broad if the causative pathogen is susceptible to narrower-spectrum agents or if the risk of antimicrobial resistance (AMR) has been overestimated. Excessive use of broad-spectrum antimicrobial agents may contribute to adverse drug effects, healthcare-associated infections, antimicrobial resistance, and increased healthcare costs (24, 29, 30).

Timely source control and subsequent tailoring of antimicrobial therapy based on microbiological findings are therefore key components of contemporary sepsis management (22). Microbiological diagnostics play a central role in identifying the causative pathogen and its susceptibility profile, thereby enabling escalation, de-escalation, or discontinuation of antimicrobial therapy (22, 29). In patients receiving inadequate empirical treatment, rapid microbiological results may facilitate earlier initiation of active therapy and reduce the time to appropriate antimicrobial treatment, which has been associated with improved outcomes in patients with bloodstream infections (23, 26). Equally important, microbiological findings, including negative results, support antimicrobial stewardship by guiding narrowing, shortening, or discontinuation of therapy and reducing unnecessary antimicrobial exposure (29, 30).

Antimicrobial resistance

Antimicrobial resistance (AMR) arises through genetic mutation and acquisition of resistance determinants, including horizontal gene transfer between bacteria, and is subsequently amplified by antimicrobial selection pressure (31). AMR is not a new phenomenon, and resistance has repeatedly emerged shortly after the introduction

of new antimicrobial agents (31). However, its global spread and clinical impact have increased substantially over recent decades, largely driven by extensive antimicrobial use in human and veterinary medicine, as well as in agriculture. As a result, AMR is now widely recognised as one of the greatest threats to global public health and is currently among the leading causes of death worldwide (32, 33).

In a systematic analysis published in *The Lancet*, bacterial AMR was estimated to have directly caused 1.27 million deaths and contributed to nearly 5 million deaths globally in 2019 (32). Among infectious syndromes, lower respiratory tract infections contributed the greatest burden of AMR-associated mortality, followed by bloodstream infections and intra-abdominal infections (32). The highest numbers of resistance-associated deaths were attributed to infections caused by *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (32).

Of particular concern is the increasing prevalence of multidrug-resistant pathogens among many of these clinically important organisms, making infections increasingly difficult to treat and threatening the safety of modern healthcare, including major surgery, transplantation, neonatal intensive care, cancer therapy, and other forms of immunosuppressive treatment (32, 33).

Addressing this challenge requires prudent antimicrobial use, effective infection prevention measures, surveillance programmes, the development of novel antimicrobial agents, and strengthened public health strategies (32, 33). At the same time, the increasing prevalence of multidrug-resistant pathogens reduces the likelihood of effective empirical therapy and may delay active treatment, thereby increasing the need for rapid pathogen identification and susceptibility testing (23, 25, 26).

Blood culture diagnostics

Microbiological diagnostics are fundamental to the management of bloodstream infections and sepsis, providing information that may support source identification, source control, and antimicrobial

treatment decisions. Despite recent advances in molecular diagnostics, blood culture remains the reference standard for the detection of bacteraemia and fungaemia and serves as the cornerstone of microbiological diagnosis in patients with suspected bloodstream infection (12). The increasing prevalence of AMR and the limitations of empirical antimicrobial therapy further underscore the importance of obtaining timely microbiological information. By enabling pathogen identification and antimicrobial susceptibility testing (AST), blood cultures play a central role in the transition from empirical to targeted antimicrobial therapy. However, blood culture diagnostics are inherently time-consuming, and clinically actionable results are often unavailable during the critical early phase of infection management (22, 29).

Principles of blood culture diagnostics

Blood culture sensitivity is strongly influenced by the volume of blood cultured. Current Swedish and international recommendations advocate 40-60 mL of blood per blood culture episode in adults, corresponding to two to three blood culture sets. Each set consists of one aerobic and one anaerobic bottle, with a target volume of approximately 10 mL per bottle. Larger blood volumes may be considered in conditions associated with low-grade or intermittent bacteraemia, such as infective endocarditis or candidaemia (12, 34, 35).

Blood cultures should be collected from a peripheral vein following meticulous skin disinfection. In patients with suspected catheter-related bloodstream infection, additional cultures should be obtained from the intravascular catheter to allow comparison with peripheral cultures (12).

Traditionally, blood culture collection has involved obtaining blood from multiple venipuncture sites and, in some cases, at different time points. While this multi-sampling strategy remains recommended in some countries, several contemporary guidelines, including Swedish recommendations, advocate a single-sampling strategy in which all

blood culture bottles are collected during a single sampling episode from the same venipuncture site (34). Studies have demonstrated that this approach increases the likelihood of obtaining the recommended total blood volume, reduces contamination rates by minimising the number of skin punctures, and simplifies the sampling procedure without compromising diagnostic sensitivity (36-38).

The aerobic bottle is typically inoculated before the anaerobic bottle to minimise the introduction of air into the anaerobic culture medium and thereby preserve optimal conditions for the recovery of anaerobic bacteria. Several national guidelines also recommend diversion of the initial portion of collected blood before inoculation of blood culture bottles to reduce contamination by skin flora and tissue fragments introduced during venipuncture (34, 39, 40).

For optimal diagnostic sensitivity, blood cultures should be obtained before initiation of antimicrobial therapy whenever clinically feasible. Although contemporary blood culture bottles contain resin-based media designed to adsorb antimicrobial agents, prior antibiotic exposure may still substantially reduce pathogen recovery due to incomplete antimicrobial neutralisation and reduced microbial viability before sampling (12, 41, 42)

Following collection, blood culture bottles are loaded into automated incubation systems, where they are continuously monitored for microbial growth. Growth is detected through repeated measurements of carbon dioxide (CO_2) production resulting from microbial metabolism using colorimetric or fluorescent sensors located at the base of each bottle. When predefined thresholds are exceeded, the instrument generates a positive signal and the bottle is removed for further processing (12). In routine practice, blood culture bottles are generally incubated for five days before being reported as negative, although extended incubation may be considered in selected clinical situations involving slow-growing or fastidious pathogens, including infections associated with prosthetic intravascular material, fungaemia, tularaemia, and brucellosis (34).

Processing of positive blood cultures

Following detection of microbial growth, blood culture bottles undergo a series of laboratory procedures aimed at providing clinically actionable information. Traditionally, Gram staining has represented the first diagnostic step following blood culture positivity. Information on Gram reaction and morphology provides early clues to the identity of the causative organism and may guide initial antimicrobial management. Positive blood cultures are subsequently processed to enable species identification and antimicrobial susceptibility testing (AST), typically following subculture on solid media. Conventional species identification and susceptibility testing usually require subculture and overnight growth, delaying complete microbiological results by approximately 18-24 hours after blood culture positivity. However, both species identification and susceptibility testing are increasingly performed directly from positive blood culture bottles using rapid diagnostic methods. Consequently, species identification may in some settings become available before, or at the same time as, conventional Gram stain results.

To guide clinical decision-making, microbiological information is communicated as results become available, typically in a stepwise manner, progressing from preliminary findings such as Gram stain results to species identification and eventually antimicrobial susceptibility data. Depending on local laboratory routines, results may be reported electronically, communicated by telephone, or both (34, 43).

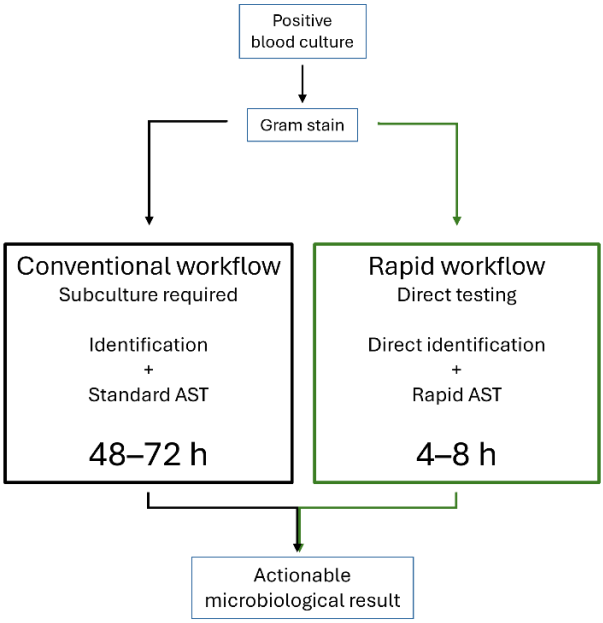


Figure 1. Simplified comparison of conventional and rapid diagnostic workflows following blood culture positivity. Elimination of the subculture step allows direct identification and rapid antimicrobial susceptibility testing (AST), reducing the time to actionable microbiological results.

Species identification

Species identification has traditionally relied on subculture of positive blood cultures followed by phenotypic characterisation and biochemical identification methods, including semi-automated systems (44). The introduction of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) represented a major advance by enabling rapid, accurate, and cost-effective identification of microorganisms from cultured colonies (43, 45). Unlike conventional biochemical methods, which rely on phenotypic and metabolic characteristics, MALDI-TOF MS identifies microorganisms by analysing species-specific protein spectra and comparing them with reference databases (45). Following its widespread adoption around 2010, MALDI-TOF MS substantially

reduced the time required for species identification and became the routine method in many CMLs. Despite these advances, conventional species identification workflows remain dependent on subculture and colony growth following blood culture positivity, thereby delaying clinically actionable identification results (43, 45).

Rapid species identification methods

To overcome the delays associated with subculture-dependent identification, several methods enabling earlier pathogen identification have been developed. Early approaches included fluorescence in situ hybridisation (FISH) and peptide nucleic acid FISH (PNA-FISH), which use fluorescent probes targeting species-specific nucleic acid sequences, as well as antigen detection methods, agglutination tests, and species-specific polymerase chain reaction (PCR) assays (44, 46).

Subsequent developments focused on shortening or bypassing the need for subculture. These include proteomic approaches, most notably direct MALDI-TOF MS, and molecular approaches such as multiplex PCR panels and hybridisation-based assays capable of detecting predefined nucleic acid targets and selected AMR genes (43). Initially, these approaches relied on MALDI-TOF MS performed on short-incubated colonies, but later evolved into methods capable of direct identification from positive blood cultures (43, 45).

Other approaches seek to bypass blood culture entirely through direct analysis of whole-blood specimens. These include magnetic resonance-based technologies and sequencing-based approaches, including 16S rRNA gene sequencing and metagenomic sequencing (47). While magnetic resonance-based methods combine nucleic acid amplification with magnetic nanoparticle-based detection and T2 magnetic resonance technology, sequencing methods identify microorganisms through analysis of microbial genetic material and are not limited to predefined species targets (47).

Direct MALDI-TOF MS

Among currently available methods for direct identification from positive blood cultures, direct MALDI-TOF MS is one of the most widely implemented approaches in CMLs due to the widespread availability of MALDI-TOF MS instrumentation and its straightforward integration into existing diagnostic workflows (48, 49). However, for direct MALDI-TOF MS analysis, microorganisms must first be separated from blood culture broth and concentrated into a purified pellet suitable for analysis. Numerous preparation protocols have therefore been developed, ranging from simple centrifugation- and filtration-based in-house procedures to commercial extraction kits (50, 51). Despite differences in complexity, cost, hands-on time, and performance, all aim to generate material suitable for reliable MALDI-TOF MS identification. Reported identification rates vary according to both the preparation protocol used and the microorganisms present. In general, direct MALDI-TOF MS performs best for Gram-negative bacteria, whereas lower identification rates have been reported for Gram-positive bacteria, yeasts, and polymicrobial blood cultures. Reduced performance is partly related to differences in cell wall structure affecting protein extraction, low microbial biomass, and the challenges associated with analysing mixed microbial populations (52-54).

Antimicrobial susceptibility testing (AST)

Antimicrobial susceptibility testing (AST) provides essential information for guiding targeted antimicrobial therapy and can broadly be divided into phenotypic and genotypic approaches.

Phenotypic antimicrobial susceptibility testing

Phenotypic AST assesses the ability of a microorganism to grow in the presence of antimicrobial agents and thereby measures the expression of susceptibility or resistance. Because it directly evaluates the overall effect of antimicrobial exposure on bacterial growth, regardless of the

underlying resistance mechanism, phenotypic AST remains the reference standard for susceptibility determination and the cornerstone of clinical susceptibility testing.

Bacterial resistance may be intrinsic or acquired and can arise through several mechanisms, including enzymatic antimicrobial degradation, modification of antimicrobial targets, reduced cell permeability, and active efflux of antimicrobial agents (55). The principal mechanisms of resistance are summarised in Figure 2.

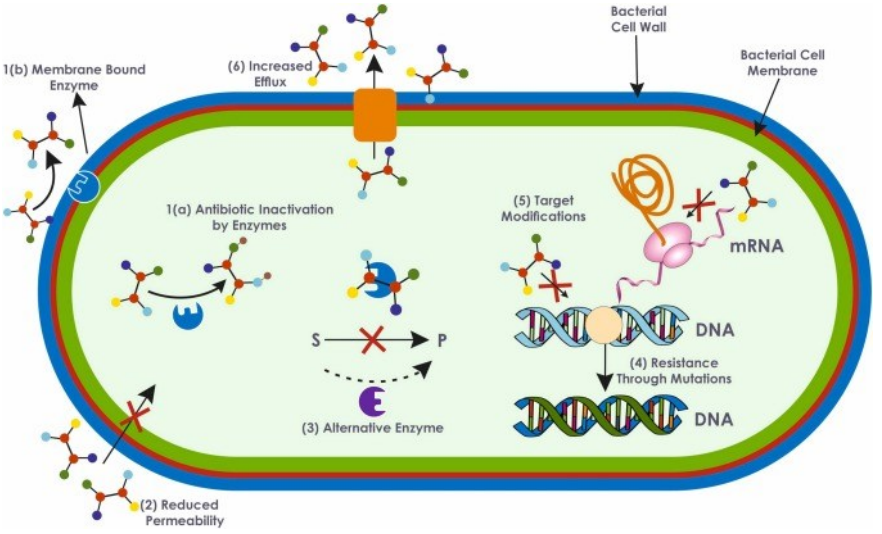


Figure 2. Major mechanisms of bacterial antimicrobial resistance. Resistance may arise through enzymatic antibiotic inactivation, reduced cellular permeability, active efflux, target modification, alternative metabolic pathways, or genetic mutations. *Adapted from Baweja R, Singh M, Shukla S, et al. Antimicrobial resistance: Mechanism, causes, prevention and societal impact. The Microbe. 2025. Licensed under CC BY 4.0.*

Minimum inhibitory concentration

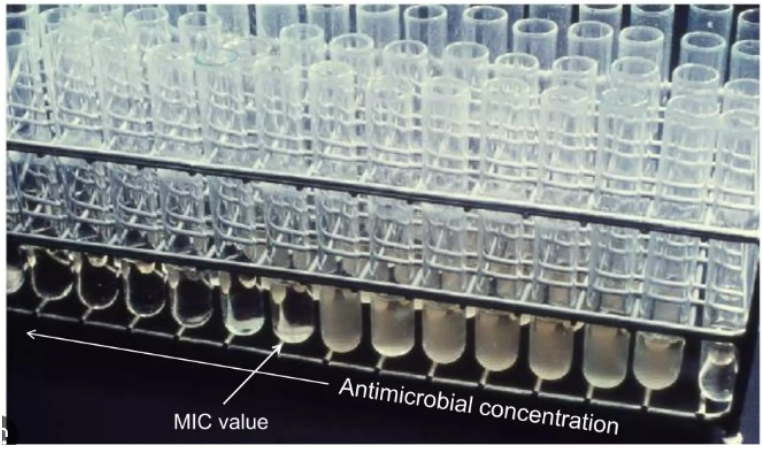
The impact of resistance mechanisms on antimicrobial susceptibility can be quantified through determination of the minimum inhibitory concentration (MIC), which corresponds to the lowest concentration

of an antimicrobial agent capable of inhibiting visible bacterial growth in vitro (56).

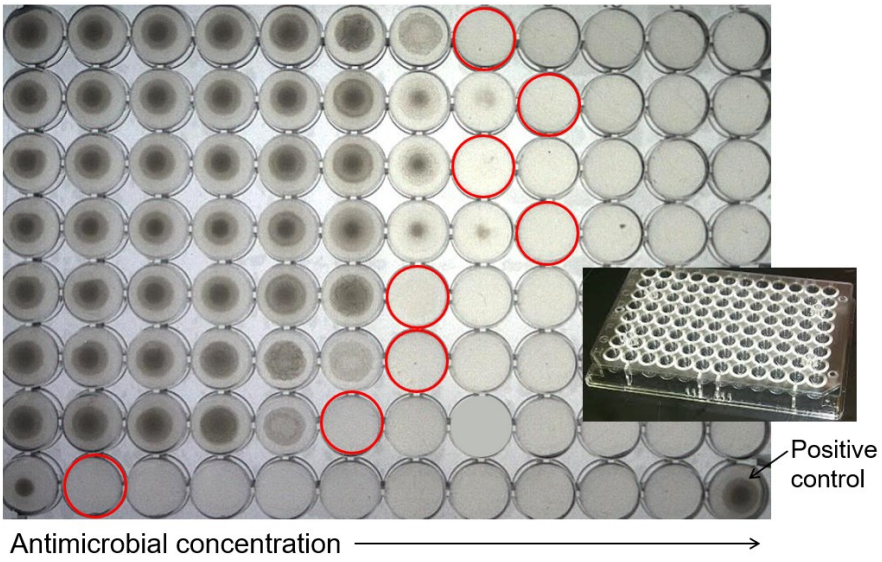
MIC determination is performed under standardised testing conditions using a defined bacterial inoculum and incubation protocol. Broth microdilution (BMD) is generally considered the reference method for AST and forms the basis for evaluation of other susceptibility testing methods (56, 57). In BMD, a standardised inoculum prepared from colonies obtained from a pure culture is added to wells containing serial two-fold dilutions of antimicrobial agents. Following the recommended incubation period, usually 16-20 h, bacterial growth is assessed and the MIC is defined as the lowest antimicrobial concentration that inhibits visible growth. As antimicrobial agents are tested in serial two-fold dilutions, MIC values are expressed on a logarithmic scale, where each dilution step represents a doubling or halving of the antimicrobial concentration. Given the inherent variability of MIC determination, minor differences of one dilution step are generally considered to fall within the expected methodological variability of the test (57). Therefore, small differences between MIC values do not necessarily reflect true biological differences in antimicrobial susceptibility.

While the original macrodilution method used serial antimicrobial dilutions in test tubes, contemporary AST is almost exclusively performed using broth microdilution in microtitre plates. The principle of MIC determination by broth dilution methods is illustrated in Figure 3.

Figure 3. Determination of minimum inhibitory concentration (MIC) by broth dilution methods.



A) Principle of broth macrodilution testing. Bacterial growth is visible at antimicrobial concentrations below the MIC, whereas no growth is observed at and above the MIC. *Reproduced with permission from Gunnar Kahlmeter*.



B) Example of broth microdilution (BMD). Antimicrobial concentrations increase from left to right. The MIC is defined as the lowest antimicrobial concentration without visible growth; example MIC endpoints are indicated by red circles. The inset shows a standard 96-well microtitre plate used for BMD testing. *Adapted from images kindly provided by the EUCAST Development Laboratory (EDL).*

MIC distributions, ECOFFs and PK/PD breakpoints

When MIC values from large collections of isolates belonging to a specific species-antimicrobial combination are plotted, characteristic MIC distributions can be generated. Typically, isolates lacking acquired resistance mechanisms cluster within a relatively narrow MIC range, forming the so-called wild-type (WT) population. The shape and range of this distribution primarily reflect the natural variability in susceptibility within the species when tested under standardised conditions (58, 59). Analysis of MIC distributions enables identification of the upper limit of the WT population. This value is referred to as the epidemiological cut-off value (ECOFF) and represents the highest MIC expected among isolates lacking phenotypically detectable acquired resistance mechanisms (59, 60). ECOFF determination is based on statistical analysis of MIC distributions combined with expert assessment of the observed data (59, 60). Isolates with MIC values above the ECOFF are classified as non-wild-type (non-WT), suggesting the presence of an acquired resistance mechanism (58, 59) (Figure 4).

The distinction between WT and non-WT populations provided by ECOFFs is valuable for AMR surveillance and facilitates identification of isolates harbouring acquired resistance mechanisms, including emerging or previously unrecognised mechanisms associated with reduced susceptibility. ECOFFs may also serve as screening thresholds for specific resistance determinants and constitute an important microbiological component in the establishment and revision of clinical breakpoints (61, 62). Importantly, ECOFFs are microbiological rather than clinical parameters. They define the upper limit of the WT MIC distribution and do not account for antimicrobial exposure, dosing regimens, or clinical outcome data. Consequently, WT isolates may not necessarily be clinically susceptible, and ECOFFs should not be interpreted as clinical breakpoints or direct predictors of treatment success or failure (56, 58, 60, 63).

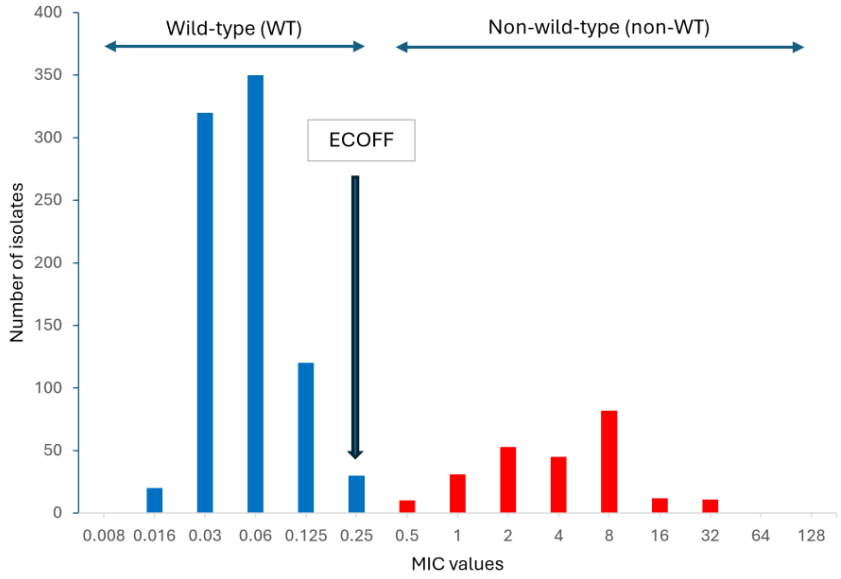


Figure 4. Hypothetical MIC distribution illustrating wild-type (WT) and non-wild-type (non-WT) populations. The epidemiological cut-off value (ECOFF) represents the upper limit of the WT population.

The establishment of a clinical breakpoint for a specific antimicrobial agent and bacterial species is a multidisciplinary process requiring integration of microbiological, pharmacological and clinical data. Key considerations include MIC distributions and ECOFFs for the target species, pharmacokinetic (PK) properties, pharmacodynamic (PD) relationships, approved dosing regimens, clinical outcome data, and known resistance mechanisms associated with reduced susceptibility (56, 62-64).

Pharmacokinetics describes how antimicrobial concentrations change in the body over time as a result of drug absorption, distribution, metabolism and elimination, whereas pharmacodynamics relates antimicrobial exposure to antibacterial activity and describes how increasing drug concentrations influence bacterial growth inhibition or killing (64). In clinical breakpoint setting, PK/PD analyses are used to identify the highest MIC for which an approved dosing regimen is expected to provide a high probability of antibacterial efficacy. Depending on the antimicrobial class,

efficacy is best predicted by different PK/PD indices, such as the ratio between the area under the concentration-time curve and MIC (AUC/MIC), the peak concentration-to-MIC ratio (Cmax/MIC), or the proportion of the dosing interval during which free drug concentrations exceed the MIC (fT>MIC). Experimental infection models and clinical studies are used both to identify the PK/PD index that best predicts efficacy and to define target values for that index associated with antibacterial efficacy (64).

For example, a specific proportion of the dosing interval with concentrations above the MIC (fT>MIC) or a defined AUC/MIC ratio may be required to achieve the desired antibacterial effect. Pharmacokinetic data from human studies are then used to characterise variability in antimicrobial exposure within the patient population. Using these data, Monte Carlo simulations are performed to model drug exposure in large numbers of virtual patients, taking interindividual pharmacokinetic variability into account. This allows estimation of the probability that a given dosing regimen will achieve the predefined PK/PD target across a range of MIC values. This probability, referred to as the probability of target attainment (PTA), generally decreases as MIC values increase, reflecting the greater antimicrobial exposure required to inhibit less susceptible organisms (64).

The highest MIC for which approved dosing regimens achieve the PK/PD target with an acceptable probability of target attainment represents a key component in clinical breakpoint setting (62-64). However, PK/PD considerations alone are insufficient for breakpoint determination. Proposed breakpoints must also be evaluated in relation to MIC distributions and ECOFFs, as well as available clinical outcome data and known resistance mechanisms. As a general principle, clinical breakpoints should encompass the WT population and avoid dividing a microbiologically homogeneous WT distribution, as this could result in microbiologically similar WT isolates being assigned different susceptibility categories (58, 62, 63).

Proposed breakpoint candidates are subsequently evaluated using clinical outcome data, ideally from studies in which MIC values for the causative pathogen are known. Such data are used to assess whether

treatment outcomes correspond to predictions derived from PK/PD analyses and may support, refine, or occasionally challenge breakpoint proposals (56, 63, 64).

In addition to PK/PD and clinical outcome data, information on resistance mechanisms is an important component of breakpoint setting. Known resistance mechanisms, their associated MIC distributions, and their clinical significance are evaluated to ensure that clinically relevant resistance can be reliably detected by the proposed breakpoint. As new resistance mechanisms emerge and additional clinical and microbiological data become available, previously established breakpoints may require revision (56, 62, 63).

Clinical breakpoints and susceptibility categories

In Europe, clinical breakpoints and susceptibility categories are defined and maintained by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Following evaluation of microbiological, pharmacological and clinical data, proposed breakpoints for new antimicrobial agents are submitted to the European Medicines Agency (EMA) and the Committee for Medicinal Products for Human Use (CHMP) for regulatory consideration prior to inclusion in the EUCAST breakpoint tables (62, 65, 66).

Clinical breakpoints are used to categorise isolates as susceptible (S), susceptible, increased exposure (I), or resistant (R). Susceptible isolates are considered likely to respond to treatment at standard antimicrobial exposure, whereas isolates categorised as susceptible, increased exposure are likely to respond when antimicrobial exposure is increased. Resistant isolates are associated with a low probability of therapeutic success. For some species-antimicrobial combinations, insufficient evidence is available to establish clinical breakpoints (IE). For others, susceptibility testing is not recommended ("–") (66).

For selected species-antimicrobial combinations, EUCAST defines an Area of Technical Uncertainty (ATU). ATUs are introduced when technical limitations of susceptibility testing make reliable discrimination between susceptibility categories difficult. Results within an ATU indicate that additional testing, confirmatory methods,

or careful interpretation may be warranted before a final susceptibility category is assigned (67).

Clinical breakpoints, susceptibility categories, ATUs, and interpretive guidance are published and regularly updated in the EUCAST breakpoint tables (66).

Disk diffusion antimicrobial susceptibility testing

Although broth microdilution (BMD) is considered the reference method for AST, its labour-intensive nature limits its use in routine CMLs. Therefore, standardised disk diffusion methods have been developed and calibrated against reference MIC methods, providing a simple, inexpensive, reproducible, and widely accessible alternative for routine susceptibility testing (68).

In disk diffusion testing, colonies obtained from a pure culture are used to prepare a standardised bacterial inoculum, which is spread on an appropriate agar medium and exposed to paper disks containing predefined amounts of antimicrobial agents. During incubation, the antimicrobial agent diffuses radially into the agar, generating a concentration gradient that inhibits bacterial growth in a concentration-dependent manner. Following the recommended incubation period of 16-20 h, the diameter of the resulting inhibition zone is measured.

To enable categorisation into the clinical susceptibility categories S, I, and R, species-specific zone diameter breakpoints have been derived and calibrated against the corresponding clinical MIC breakpoints (68, 69). Larger inhibition zones generally correspond to lower MIC values, whereas smaller zones correlate with higher MIC values (68).

Establishment of zone diameter breakpoints requires testing of large collections of clinical isolates using both disk diffusion and reference MIC methods. Resulting MIC values and inhibition zone diameters are analysed using MIC-zone diameter scattergrams and distribution analyses. Analysis of MIC and zone diameter distributions, MIC-zone diameter correlations, and ECOFFs is used to derive zone diameter breakpoints corresponding to the clinical MIC

breakpoints (69, 70). An example of disk diffusion breakpoint calibration based on MIC-zone diameter scattergram analysis is shown in Figure 5.

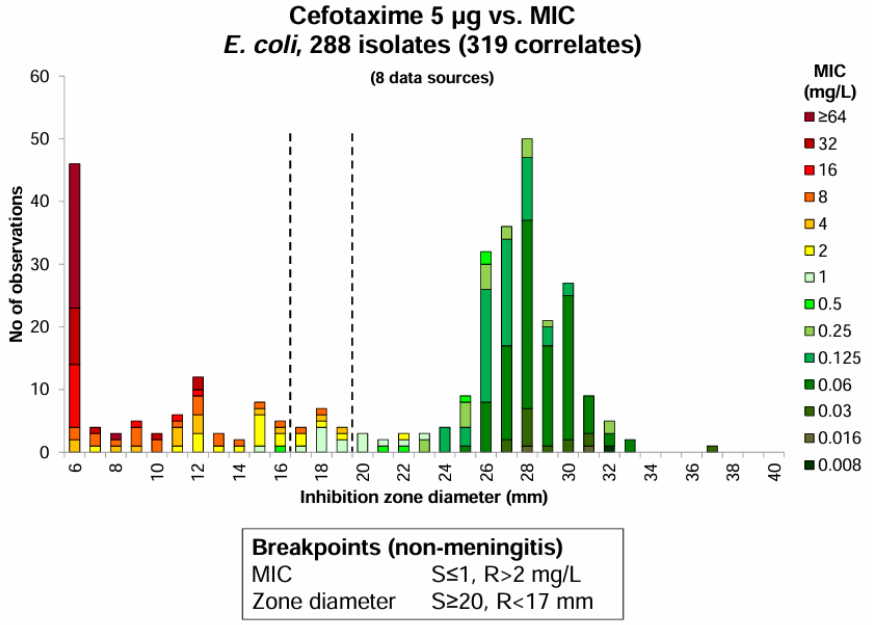


Figure 5. Relationship between inhibition zone diameters and reference MIC values for cefotaxime in *Escherichia coli*, illustrating calibration of disk diffusion zone diameter breakpoints against clinical MIC breakpoints. *Adapted from EUCAST calibration data for Enterobacterales (71).*

The disk content is selected to provide optimal separation of WT and non-WT populations while maintaining accurate categorisation according to the clinical breakpoints (70). As for MIC breakpoints, particular emphasis is placed on avoiding division of the WT population (62, 63, 68, 69). Because zone diameters represent a continuous measurement, disk diffusion often provides higher resolution within the WT population than MIC determination, which is restricted to discrete two-fold dilution steps. However, the relationship between inhibition zone diameters and MIC values is not strictly one-to-one, as illustrated in Figure 5, and zone diameters

therefore cannot be directly translated into exact MIC values. Disk diffusion is consequently calibrated primarily to the final susceptibility categories S, I, and R rather than to individual MIC values (68-70).

Quality control (QC)

To ensure reliable and reproducible susceptibility testing, EUCAST provides quality control recommendations based on designated quality control (QC) strains. These strains are tested regularly together with the media, antimicrobial agents, and test procedures used in routine susceptibility testing. Measured MIC values or inhibition zone diameters are expected to fall within predefined target ranges, thereby verifying correct test performance and supporting the validity of patient results (72, 73).

Genotypic susceptibility testing

Genotypic AST relies on detection of genetic determinants associated with AMR rather than assessment of bacterial growth. Molecular AST methods include targeted PCR assays, multiplex molecular panels, microarrays, and sequencing-based approaches (74), while untargeted metagenomic approaches are under development (75). In simplified terms, phenotypic testing reveals whether an isolate is resistant, whereas genotypic testing may help explain why.

A major advantage of genotypic methods is their ability to shorten the time to microbiological results by detecting resistance determinants directly in positive blood cultures or, in some cases, directly in clinical specimens without the need for prolonged cultivation (74, 76).
Direct genotypic testing may also enable detection of fastidious or non-cultivable microorganisms and requires smaller sample volumes than conventional blood culture, which may be advantageous in settings such as paediatric care. In addition, genotypic methods do not necessarily require viable microorganisms and may therefore be less affected by prior antimicrobial therapy (74, 76).

Despite these advantages, translating genotypic findings into clinically meaningful susceptibility predictions remains challenging. Detection of a resistance determinant does not necessarily predict phenotypic resistance or the precise susceptibility category. Furthermore, compared with phenotypic AST, which provides quantitative susceptibility data for multiple antimicrobial agents simultaneously, genotypic methods are generally limited to selected resistance determinants and provide less information regarding the extent of resistance (76, 77).

Genotypic methods are restricted to known resistance-associated genes and mutations and may therefore fail to detect novel or non-targeted resistance determinants (77, 78). Phenotypic resistance may also arise through interactions between multiple genetic determinants, regulatory mechanisms, and altered gene expression that are difficult to predict from genotypic data alone (75, 78).

Additional challenges arise when genotypic methods are applied directly to clinical specimens. Resistance genes detected in polymicrobial or non-sterile samples may not necessarily originate from the causative pathogen, making attribution of resistance determinants to the infecting organism difficult, while low microbial biomass often necessitates amplification or enrichment procedures, thereby offsetting some of the time-saving advantages of direct analysis (76).

Overall, while detection of selected resistance determinants may reliably predict resistance to specific antimicrobial agents, such as *mecA/mecC* in *Staphylococcus aureus* or *vanA/vanB* in enterococci, genotypic methods generally provide less certain information regarding which antimicrobial agents are likely to be clinically effective. Consequently, genotypic methods currently complement rather than replace phenotypic susceptibility testing, which remains the reference standard for clinical AST (74, 77, 78).

Rapid antimicrobial susceptibility testing (RAST)

Despite major advances in rapid species identification and molecular detection of resistance determinants, clinically relevant AST remains primarily dependent on phenotypic methods. Conventional testing generally requires subculture followed by overnight incubation, delaying AST results by 18-24 h after species identification. Consequently, considerable efforts have been directed towards shortening turnaround times (TATs). Several approaches have been evaluated to reduce or eliminate the need for subculture, including direct testing from positive blood culture bottles using disk diffusion or gradient diffusion methods (79-81), as well as direct inoculation into automated phenotypic identification and susceptibility testing systems (82, 83).

In parallel, numerous studies have investigated whether AST results can be obtained after shortened incubation periods. Early reading strategies have been evaluated using both standardised inocula prepared from isolated colonies and direct inoculation from positive blood cultures (79, 84-88). Similar approaches have also been explored for urine specimens, which are of particular interest as urinary tract infections represent a common source of bloodstream infection and sepsis. Similar to positive blood cultures, the often high bacterial burden may facilitate direct testing without prior isolation of colonies (89-91).

However, a recurring challenge in studies evaluating early reading of conventional growth-based AST methods has been that breakpoints established for standard incubation times do not always perform adequately when applied to earlier readouts. Specifically, separation of WT and non-WT populations may be insufficient, increasing the risk of misclassification. As bacterial responses to antimicrobial exposure continue to evolve during incubation, early zone diameters or MIC values may not fully correspond to final results, increasing the risk of categorical errors (84-87).

To overcome limitations associated with both subculture-dependent workflows and conventional growth-based endpoints, several

dedicated rapid phenotypic platforms have been developed and commercialised. Rather than relying on visible bacterial growth, these systems detect early biological responses to antimicrobial exposure. Depending on the platform, susceptibility assessment may be based on automated microscopy, microfluidics, fluorescence-based viability measurements, rapid broth microdilution, or metabolic sensing approaches. By capturing early phenotypic responses to antimicrobial exposure, including changes in growth, morphology, physiology, or metabolism, these systems can provide AST results directly from positive blood culture bottles within a few hours (76, 92, 93). Although these technologies have demonstrated substantial reductions in time to result, implementation often requires dedicated instrumentation, specialised consumables, and modifications of established laboratory workflows. In addition, throughput may be limited compared with conventional laboratory systems (76, 92, 93)

Emerging technologies are increasingly moving beyond conventional growth measurements to focus on parameters such as cellular metabolism, cell stress responses, biomass changes, nanomotion, electrical properties, and single-cell behaviour. These approaches are discussed further in Future Perspectives (93).

EUCAST RAST

Building on previously described direct susceptibility testing and early-reading approaches, EUCAST explored the feasibility of developing a standardised RAST methodology that could be implemented using existing laboratory infrastructure and independently of blood culture system manufacturer. This resulted in the development of EUCAST rapid antimicrobial susceptibility testing (RAST), a standardised disk diffusion method performed directly from positive blood culture bottles (94).

The method was developed to provide reliable susceptibility results for the most common bloodstream infection pathogens within the same working day. To address the limitations observed in previous direct testing and early-reading studies (84-87), EUCAST developed

species-specific and incubation time-specific zone diameter breakpoints for readings performed after 4, 6, and 8 h of incubation. These breakpoints were specifically calibrated for early reading to improve separation of WT and non-WT populations compared with conventional disk diffusion breakpoints.

To further reduce the risk of susceptibility misclassification resulting from overlap between WT and non-WT populations, EUCAST applied the concept of an Area of Technical Uncertainty (ATU) to RAST. In addition to addressing the incomplete separation of susceptibility populations observed at early reading, the ATU accommodates variability associated with the non-standardised inoculum and other methodological differences inherent to direct testing from positive blood cultures and shortened incubation times. Results falling within the ATU are not assigned a susceptibility category and require either additional incubation or testing by a conventional susceptibility method. Consequently, EUCAST RAST allows categorical interpretation only as susceptible (S) or resistant (R). Isolates falling within the ATU remain uninterpretable at that reading time and may require further testing before a final result can be reported (94).

The development study demonstrated that rapidly growing species such as *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were frequently reportable after 4 h of incubation, whereas *Pseudomonas aeruginosa* generally required at least 6 h before reliable interpretation was possible. Depending on the species, the proportion of interpretable results ranged from approximately 51-80% after 4 h of incubation and increased to 75-85% after 8 h. Across the evaluated species and incubation times, categorical agreement (CA) ranged from 96.7% to 100% (94).

The preliminary breakpoints and overall performance of the proposed method were subsequently evaluated in a large multicentre validation study involving 55 CMLs across northern and southern Europe (95). The study demonstrated that the method could be reproducibly implemented across different laboratories while maintaining high CA with the reference method. As in the

development study, the proportion of interpretable results increased with incubation time. Importantly, the multicentre data enabled refinement of the preliminary breakpoints to account for inter-laboratory variability. At the same time, the revised breakpoints increased the proportion of reportable results and reduced the number of results falling within the ATU. The findings of the validation study supported standardisation of the method by EUCAST, enabling its publication as a freely available methodology that could be adopted for routine use in CMLs (95).

Since the initial development and validation studies, the range of species and antimicrobials covered by EUCAST RAST has gradually expanded. Species-specific breakpoints are currently available for several of the most important bloodstream infection pathogens, including *Escherichia coli, Klebsiella pneumoniae, Salmonella enterica, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium* and *Streptococcus pneumoniae*. In addition, the original reading times of 4, 6 and 8 h have been complemented by species-specific interpretive criteria for incubation periods of 16-20 h (96, 97).

Beyond the original development and multicentre validation studies, EUCAST RAST has been extensively evaluated by independent groups across diverse laboratory settings and study populations, with reported CA typically ranging from 95% to 100% (98-104). High proportions of ATU results have consistently been reported for certain antibiotic-pathogen combinations, most notably piperacillin-tazobactam at early reading times, although the frequency of such results generally decreases with increasing incubation time. False susceptible results have generally occurred at low frequencies (98-104). The method has also been evaluated in collections enriched with ESBL- and carbapenemase-producing isolates (101, 103) as well as in workflows incorporating automated incubation and digital image acquisition (100, 102). Furthermore, clinical implementation studies have demonstrated reduced turnaround times and earlier optimisation of antimicrobial therapy (99, 104, 105).

Total blood culture turnaround time

The blood culture diagnostic pathway is commonly divided into pre-analytical, analytical and post-analytical phases. Collectively, these phases determine the total blood culture turnaround time (TAT), which in this thesis refers to the time from blood culture collection to clinically actionable treatment decisions based on microbiological results.

Diagnostic TAT refers to the interval from blood culture collection until microbiological results become available for clinical use and thus comprises the pre-analytical and analytical phases of the diagnostic pathway.

Each of the pre-analytical, analytical, and post-analytical phases is associated with inherent delays arising from biological, technical, and organisational factors. Consequently, turnaround times are influenced by multiple processes within and outside the microbiology laboratory (43, 106). For the purposes of this thesis, the blood culture diagnostic phases are defined as follows:

Pre-analytical phase

The pre-analytical phase comprises all steps from blood culture collection to blood culture positivity. This includes transport of blood culture bottles, loading into a blood culture incubator, and subsequent incubation until microbial growth is detected (43, 106).

Analytical phase

The analytical phase begins when a blood culture bottle signals positive and encompasses all laboratory procedures performed to identify the causative microorganism and determine its antimicrobial susceptibility, culminating in the availability of microbiological results for clinical use (43, 106).

Post-analytical phase

The post-analytical phase begins when microbiological results become available to the treating clinician and comprises the integration of these findings into clinical decision-making, including modification of antimicrobial therapy when indicated (43, 106).

Sources of diagnostic delay

The clinical value of blood culture diagnostics depends not only on the accuracy of microbiological results but also on how rapidly those results become available and are incorporated into clinical decision-making. Consequently, reducing diagnostic delays has become an important objective in efforts to optimise the management of bloodstream infections and sepsis (22). The relative contribution of different sources of delay varies between institutions and is influenced by local routines, laboratory organisation and available diagnostic technologies (12, 106).

The pre-analytical phase constitutes the longest component of the blood culture diagnostic pathway. As conventional blood culture diagnostics rely on microbial replication until detectable growth is achieved, a substantial proportion of this phase is biologically determined and cannot be eliminated by current culture-based methods (107). Time to positivity (TTP), defined as the time from incubation to blood culture positivity, varies between microorganisms. Many common bloodstream pathogens, including Enterobacterales and streptococci, are typically detected within approximately 10-15 hours of incubation. In contrast *Staphylococcus aureus*, *Pseudomonas aeruginosa* and some less common Gram-negative species often require several additional hours before growth is detected. Slow-growing organisms may require more than 24 hours of incubation to reach the detection threshold (108, 109).

TTP is also influenced by microbial burden, with higher inoculum concentrations generally resulting in shorter detection times. Consequently, blood volume cultured is an important determinant of both blood culture yield and TTP (12, 110). Microbial burden may

further vary according to the source and type of infection, thereby contributing to variation in TTP between patients and clinical syndromes (107-109). In addition, administration of antimicrobial therapy before blood culture collection may both reduce blood culture sensitivity and prolong TTP among cultures that remain positive (41, 42, 109).

Although the biological component of this phase is largely unavoidable, technical factors may influence the interval required for growth detection. Comparative studies have demonstrated differences in detection times between commonly used automated blood culture platforms and bottle formulations, indicating that both culture media composition and growth detection technology contribute to variability in TTP (111, 112).

Organisational factors may also substantially prolong the pre-analytical phase. The time required for blood culture bottles to reach an incubator depends on transport logistics, geographical distance to incubation facilities, and whether these are available around the clock. Delayed incubation has been associated with reduced blood culture sensitivity and prolongation of the overall diagnostic process (12, 113, 114). While microbial growth may continue before bottles are loaded into the incubation system, resulting in an apparently shorter measured TTP, delays before incubation nevertheless increase the total time to microbiological results and thereby prolong the total blood culture turnaround time (107, 115).

The analytical phase has historically been a major target for initiatives aimed at reducing delays in blood culture diagnostics, and advances in rapid species identification and AST have markedly shortened this phase (47, 116). Once a positive blood culture becomes available for analysis, a wide range of rapid methods can be applied to identify the causative pathogen and, in some cases, determine antimicrobial susceptibility within a few hours. However, diagnostic methods performed directly on whole blood remain considerably more limited and are not yet widely implemented in routine clinical practice (74).

Nevertheless, logistical and organisational factors may still contribute substantially to delay, as blood culture processing remains highly dependent on laboratory workflow and staffing (117-119). Continuous 24/7 processing of positive blood cultures remains uncommon in many healthcare systems, and blood culture bottles signalling positive outside laboratory operating hours may therefore remain unattended for prolonged periods before analysis can be initiated (48, 49, 119). Furthermore, when blood culture incubators are geographically separated from the laboratory performing downstream analyses, transport logistics may introduce additional delays before positive cultures can be processed (43, 49, 119).

Finally, delays may occur due to the review and validation of microbiological results before their release. The magnitude of such delays depends on local laboratory organisation, staffing models and reporting procedures (117, 119, 120).

Post-analytical delays occur after microbiological results become available and may arise during result communication, clinical review of findings, and the subsequent modification of antimicrobial therapy. The magnitude of these delays depends on local clinical workflows, organisation of inpatient care, and availability of antimicrobial stewardship support (121-124).

The clinical value of microbiological results depends not only on the diagnostic information generated but also on how efficiently that information is transferred to and acted upon by the treating team. Communication may occur through written laboratory reports, electronic reporting systems, and/or direct telephone notification. However, rapid availability of microbiological results does not necessarily translate into prompt modification of antimicrobial therapy (119). Delays may arise when results are communicated passively, become available outside routine clinical review periods, or reach clinicians who are unable to immediately review and act upon the information (119, 121, 122).

Consequently, rapid availability of microbiological results alone is often insufficient to ensure timely modification of antimicrobial therapy. Several studies have demonstrated that the clinical benefit of

rapid microbiological diagnostics is greatest when results are actively reviewed by clinicians with expertise in antimicrobial therapy or integrated into antimicrobial stewardship programmes, structured interventions aimed at optimising antimicrobial use (29). Such approaches facilitate interpretation of microbiological findings, support timely optimisation of antimicrobial treatment, and reduce delays between result availability and clinical action (121-125).

Despite substantial advances in blood culture diagnostics, important challenges remain regarding both the speed of microbiological testing and the translation of microbiological results into clinical action. As these challenges arise during the pre-analytical, analytical and post-analytical phases of the diagnostic pathway, further optimisation of diagnostic methods and diagnostic workflows may facilitate earlier targeted antimicrobial therapy and improve the clinical utility of blood culture diagnostics in patients with bloodstream infection and sepsis.

Hypothesis and aims

We hypothesised that microbiological diagnostics in bacteraemia can be accelerated without compromising diagnostic quality, and that optimisation of the diagnostic pathway may facilitate earlier adequate antimicrobial therapy.

The overall aim of this thesis was to improve microbiological diagnostics in patients with bacteraemia by evaluating rapid antimicrobial susceptibility testing (RAST) approaches, identifying opportunities to optimise blood culture diagnostic pathways, and assessing the clinical impact of shortened diagnostic turnaround times (TATs) on antimicrobial therapy.

The specific aims for each study were:

I. To assess the diagnostic performance, accuracy, robustness, and feasibility of the EUCAST RAST method for direct testing of positive blood cultures in routine clinical laboratories, and to refine preliminary RAST breakpoints based on data from development and validation studies.

II. To evaluate early disk diffusion zone reading for antimicrobial susceptibility testing of *Haemophilus influenzae* and *Streptococcus pneumoniae*, including the suitability of current EUCAST clinical breakpoints and Areas of Technical Uncertainty (ATUs) for interpretation of early results.

III. To characterise contemporary blood culture diagnostic practices in Nordic clinical microbiology laboratories, evaluate changes in workflows and laboratory organisation since a nationwide Swedish survey conducted in 2013, and identify opportunities for further optimisation of the blood culture diagnostic pathway.

IV. To evaluate the impact of workflow improvements on blood culture TATs and microbiological reporting, assess their potential to facilitate earlier optimisation of antimicrobial therapy, investigate the contribution of clinicians' response times to delays in initiation of adequate antimicrobial treatment, and identify remaining barriers limiting further reductions in diagnostic TATs.

Methods

General description

This thesis comprises four studies employing complementary methodological approaches to evaluate rapid antimicrobial susceptibility testing, blood culture diagnostic workflows, and the clinical impact of diagnostic optimisation. The main methodological characteristics of the included studies are summarised in Table 1, whereas detailed descriptions of study design, materials, methods, and statistical analyses are provided in the respective publications.

Study overview

Table 1. Overview of study design, materials and objectives.

Study	Design	Setting/Material	Main objective
I	Multicentre laboratory validation	55 clinical microbiology laboratories from Northern and Southern Europe; 1,151 isolates from positive blood cultures	Validation of the EUCAST RAST method from positive blood cultures
II	Laboratory method evaluation	73 *H. influenzae* and 112 *S. pneumoniae* isolates	Evaluation of early disk diffusion zone reading
III	Nordic survey	49 Nordic clinical microbiology laboratories	Characterisation of blood culture workflows
IV	Retrospective before-and-after study	Region Östergötland; 25,542 positive blood culture bottles and 463 bacteraemia episodes across three study periods	Evaluation of the clinical impact of blood culture workflow optimisation

Laboratory studies (Studies I-II)

Studies I and II were laboratory-based investigations focusing on rapid phenotypic antimicrobial susceptibility testing. Both studies were based on disk diffusion methodology and evaluated the potential

to shorten the time to susceptibility results by interpreting inhibition zones within 8 h of incubation, while maintaining acceptable diagnostic performance and reliability.

In **Study I**, 55 clinical microbiology laboratories (CMLs) from Northern and Southern Europe participated in the multicentre validation of the preliminary EUCAST rapid antimicrobial susceptibility testing (RAST) method (94). This RAST method follows standard EUCAST disk diffusion methodology (126) but incorporates adaptations required for direct testing from positive blood culture bottles, including modified incubation times and time-dependent interpretation criteria. As the method is performed directly from positive blood culture bottles, prior subculture is not required.

Participating laboratories inoculated 100-150 µL of undiluted positive blood culture broth onto Mueller-Hinton (MH) or Mueller-Hinton Fastidious (MH-F) agar, depending on the bacterial species investigated. Antimicrobial disks representing agents relevant for the treatment of bloodstream infections and sepsis were applied, and inhibition zones were read after 4, 6 and, when possible, 8 h of incubation. Zone diameter measurements were recorded and submitted together with the corresponding isolates to the EUCAST Development Laboratory (EDL) in Växjö.

At the EDL, species identification was confirmed and reference susceptibility testing was performed using standard EUCAST disk diffusion methodology (126), which served as the primary reference method for evaluation of the RAST results. Broth microdilution according to ISO 20776–1 (127), using EUCAST MH-F broth for *Streptococcus pneumoniae,* was additionally performed for a subset of isolates to assess whether RAST performance estimates were comparable when susceptibility categorisation was based on MIC values rather than disk diffusion results. The submitted RAST zone diameter measurements were interpreted using the preliminary breakpoint criteria and compared with reference susceptibility results categorised according to the contemporaneous EUCAST clinical breakpoints (128) to assess diagnostic performance. Performance measures included categorical agreement (CA), major errors (ME),

very major errors (VME) and minor errors (mE), robustness across participating laboratories, and evaluation of the preliminary breakpoint criteria.

In **Study II**, early reading of conventional disk diffusion testing was evaluated using 73 *Haemophilus influenzae* and 112 *Streptococcus pneumoniae* isolates. The isolates were selected to represent challenging susceptibility patterns, with particular emphasis on β-lactam resistance, including isolates with low-level resistance. Susceptibility testing was performed according to standard EUCAST disk diffusion methodology (129), with additional zone diameter readings performed after 6 and 8 h of incubation. For a subset of *S. pneumoniae* isolates (n=78), susceptibility testing was repeated on an alternative agar medium to assess the reproducibility of early test results. Early zone diameters were independently read by two technicians to assess inter-reader reproducibility, generating duplicate measurements for each isolate and reading time. All isolates had been characterised previously by broth microdilution and corresponding MIC values were available.

Zone diameter measurements obtained after 6 and 8 h of incubation were compared with reference susceptibility results.
MIC values were used as the primary reference method, while reference disk diffusion results were used for antimicrobials interpreted using screening criteria or requiring detection of inducible resistance mechanisms. Current EUCAST clinical breakpoints for standard disk diffusion testing were applied to the early zone diameter readings to evaluate their ability to correctly categorise isolates (130). In addition, the utility of Areas of Technical Uncertainty (ATUs) positioned in regions with limited separation between susceptible and resistant isolates was explored as a potential strategy to reduce categorical errors.

Survey study (Study III)

In **Study III**, a questionnaire consisting of 50 multiple-choice questions, supplemented by optional free-text comment fields, was

developed to investigate blood culture diagnostic practices in Nordic CMLs. The survey covered key aspects of the pre-analytical and analytical phases of the diagnostic pathway, including laboratory staffing, opening hours, placement and accessibility of blood culture incubators, species identification and antimicrobial susceptibility testing (AST) methods, reporting routines, and quality indicators such as diagnostic turnaround times (TATs). A national coordinator was appointed in each participating country to assist with survey distribution and to assess the representativeness of responding laboratories. The questionnaire was distributed to all 76 CMLs in the Nordic countries, with a response period of three weeks.

Survey responses were analysed to characterise current blood culture diagnostic services and workflows across participating Nordic laboratories. In addition, responses from Swedish laboratories were compared with those obtained in a similar nationwide survey conducted in Sweden in 2013. The survey findings were subsequently used to identify opportunities for further optimisation of blood culture diagnostic workflows.

Clinical implementation study (Study IV)

Study IV was a retrospective single-centre study conducted in Region Östergötland between 2015 and 2022. A longitudinal before-and-after design was selected to evaluate the cumulative impact of workflow interventions implemented under routine clinical conditions. Time stamps for blood culture collection, incubation start, blood culture positivity, initiation of laboratory work-up, Gram stain reporting, and species identification were extracted from the laboratory information system to reconstruct diagnostic TATs. For episodes of *Enterococcus* spp. and *Pseudomonas* spp. bacteraemia, timestamps for clinician review of microbiological results and subsequent modification of inadequate empirical antimicrobial therapy were extracted from electronic medical records.

Two major workflow interventions were introduced. The first involved decentralisation of blood culture incubation through placement of incubators at the clinical chemistry laboratories of all

regional hospitals, enabling continuous 24/7 loading of bottles into the incubation system. The second involved implementation of rapid species identification directly from positive blood cultures using MALDI-TOF MS. In addition, reporting routines were modified. Trained laboratory staff were permitted to issue preliminary Gram stain reports without prior review by a clinical microbiologist, and this practice was later extended to include preliminary species identification. These changes were considered part of the implementation context and were not evaluated as separate interventions.

Based on the timing of the major interventions, one pre-intervention period and two post-intervention periods were defined and compared.

Inclusion and exclusion criteria

All positive blood culture bottles were included in analyses of pre-analytical and analytical TATs, except for time to species identification, which was evaluated only for blood culture bottles yielding *Escherichia coli, Staphylococcus aureus, Enterococcus* spp., and *Pseudomonas* spp. Analyses of post-analytical TATs were restricted to episodes of *Enterococcus* spp. and *Pseudomonas* spp. bacteraemia. These pathogen groups were chosen to represent clinical scenarios in which species identification may have particular therapeutic relevance owing to the risk of inadequate empirical antimicrobial therapy (131, 132). For these analyses, all episodes of *Pseudomonas* bacteraemia were included. For *Enterococcus* spp., the first 50 consecutive bacteraemia episodes identified within each study-period year were included, while additional episodes were restricted to mixed *Pseudomonas-Enterococcus* bacteraemia.

For therapy-related analyses, repeated isolation of the same organism during a single hospitalisation was considered part of the same bacteraemia episode and was not analysed separately. Timestamps were obtained from the blood culture bottle in which the organism was first identified within each episode.

Patient exclusion criteria for therapy-related analyses included end-of-life palliative care, death or transfer to another hospital before availability of the first blood culture result, and admission to specialised intensive care units (ICUs) (burn, cardiothoracic, or neurosurgical intensive care units). Patients admitted to a general ICU were eligible for inclusion.

Outcome measures and definitions

The main outcome measures included TATs across the pre-analytical, analytical, and post-analytical phases of the blood culture diagnostic pathway, as well as overall time to adequate antimicrobial therapy. In addition, length of hospital stay and 30-day and 90-day all-cause mortality were assessed for episodes of *Enterococcus* spp. and *Pseudomonas* spp. bacteraemia.

Pre-analytical, analytical, and post-analytical TATs were defined as the intervals from blood culture collection to initiation of incubation, from positive blood culture signal to reporting of Gram stain or species identification results, and from microbiological result reporting to initiation of adequate antimicrobial therapy, respectively. Total TAT was defined as the interval from blood culture collection to initiation of adequate antimicrobial therapy. Fractional TATs were defined to reflect workflow components amenable to intervention. Consequently, the pre-analytical TAT comprised only the interval from blood culture collection to initiation of incubation, whereas time to positivity remained included in total TAT.

Empirical therapy was defined as the initial antimicrobial treatment regimen administered before microbiological results became available, whereas adequate therapy was defined as the first antimicrobial regimen providing adequate coverage of the identified pathogen.

Statistics

Descriptive statistics are presented as counts and percentages, means with ranges, or medians with interquartile ranges (IQR), as

appropriate. Differences in categorical variables were assessed using Fisher's exact test (Studies III and IV) or chi-square test (Study IV). Fisher's exact test was preferred when expected cell counts were small. In Study IV, differences in continuous variables across the three study periods were analysed using the Kruskal-Wallis test with Dunn's post hoc multiple comparisons test. All tests were two-sided, and p-values <0.05 were considered statistically significant.

Ethical considerations

Among the four studies included in this thesis, only Study IV involved patient data and required formal ethical approval. Studies I and II were laboratory-based investigations using bacterial isolates without access to patient identifiers, while Study III was a survey addressing organisational structures, workflows, and diagnostic practices at the laboratory level rather than individual patients. Consequently, ethical considerations in these studies were primarily related to responsible data handling and reporting rather than protection of personal health information.

Study IV was retrospective in nature and included a review of medical records. As no interventions were performed and all data were analysed in a de-identified form, the principal ethical concern was the potential intrusion into patient privacy. Owing to the retrospective study design and the use of anonymised data, informed consent was waived. Ethical approval was obtained from the Swedish Ethical Review Authority in Linköping (reference number M17-09), and the study was conducted in accordance with the principles of the Declaration of Helsinki.

Results

The studies included in this thesis were conducted over a period during which blood culture diagnostics evolved substantially. While early efforts largely focused on improving analytical turnaround times (TATs), increasing attention has gradually been directed towards optimisation of the entire diagnostic pathway, including pre-analytical and post-analytical phases. The results are therefore presented according to the main objectives of the thesis, illustrating the progression from evaluation of rapid antimicrobial susceptibility testing (RAST) methods to optimisation of the broader blood culture diagnostic process.

Rapid antimicrobial susceptibility testing

- ✓ Rapid phenotypic antimicrobial susceptibility testing (AST) methods enabled reliable susceptibility testing within a single working day for the most common bloodstream pathogens.
- ✓ Rapid phenotypic AST methods could be implemented across routine clinical microbiology laboratories without major investments in new laboratory instrumentation.

Study I included 1,151 clinical bloodstream isolates representing five major bloodstream pathogens (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus pneumoniae*) collected and tested by 55 laboratories across 11 European countries. *E. coli* accounted for approximately half of all isolates. Depending on the pathogen-antimicrobial combination, resistance rates ranged from 0% to 34%. No systematic differences in zone diameter distributions were observed according to blood culture system, agar manufacturer, or antimicrobial disk manufacturer. Furthermore, aggregated zone diameter distributions were comparable between the initial development study and the subsequent multicentre validation study. Readability after 4 h was highest for *E. coli* and *K. pneumoniae*, while *P. aeruginosa* required 6 h of incubation before zone interpretation was feasible.

Overall readability was 88%, 96%, and 99% after 4, 6, and 8 h of incubation, respectively.

Using the preliminary breakpoint criteria established in the preceding development study (94), Categorical agreement (CA) increased from 96.9% to 98.2% with prolonged incubation, while the proportion of area of technical uncertainty (ATU) results decreased from 20% to 14%. Across all readable resistant isolate-incubation time combinations, 66 VMEs (false-susceptible results; 4.0%) were observed. Fluoroquinolones accounted for a substantial proportion of these errors. Application of the revised ciprofloxacin standard disk diffusion breakpoint for Enterobacterales, introduced in the subsequent EUCAST breakpoint table (133) to improve concordance between disk diffusion and MIC categorisation, reduced the number of VMEs from 66 (4.0%) to 46 (2.8%).

Detection of clinically important resistance mechanisms, including ESBL production (n=62), carbapenemase production (n=26), and methicillin resistance in *S. aureus* (n=24), showed overall high performance, although occasional misclassifications occurred for selected isolate-antimicrobial combinations.

Among the species evaluated, *S. pneumoniae* represented the greatest challenge for early zone interpretation. In contrast to the other species, CA decreased with prolonged incubation. Except for one VME, all misclassifications were major errors (MEs; false-resistant results).

Based on the validation results, the preliminary breakpoint criteria were refined and the first official EUCAST RAST breakpoint table was introduced (134). Compared with the preliminary criteria, the revised breakpoint table substantially increased reportability by reducing the number of results falling within the ATU, while maintaining high CA (97.0%-97.8%) and similar VME rates (Table 2). In addition, interpretation rules for inducible clindamycin resistance detection in *S. aureus* and *S. pneumoniae* were refined and incorporated. The validation data also contributed to subsequent refinements of the RAST methodology, including interpretation rules for oxacillin testing in *S. pneumoniae* (135).

Table 2. Performance characteristics of the preliminary and first official EUCAST RAST breakpoint tables. The principal limitation of the preliminary breakpoint table was fluoroquinolone categorisation, predominantly due to ciprofloxacin-related VMEs.

RAST breakpoint table	Incubation time	Readability (%)	CA (%)	ATU (%)
Preliminary breakpoint table	4h	88	96.9	20
	6h	96	98.2	16
	8h	99	98.2	14
Final breakpoint table (v1.0)	4h	87	97.0	16
	6h	96	97.9	7.5
	8h	99	97.9	5.7

The *H. influenzae* and *S. pneumoniae* isolate collections in study II exhibited resistance rates ranging from 0% to 19% and 0% to 52%, respectively, depending on the antimicrobial agent evaluated. Reduced susceptibility to β-lactam agents was present in 30% of *H. influenzae* isolates and 54% of *S. pneumoniae* isolates.

Readability improved with incubation time for *H. influenzae*, from 19% after 6 h to 89% after 8 h, indicating that 8 h was the shortest feasible incubation time for early reading. Application of the contemporary EUCAST clinical breakpoints to the 8 h readings yielded a CA of 98%. All ampicillin- and cefotaxime-resistant isolates were correctly identified, and errors for non-β-lactam agents were uncommon.

For *S. pneumoniae*, readability increased from 63% after 6 h to 98% after 8 h. CA was 95% and 97% after 6 and 8 h of incubation, respectively, and the oxacillin screening breakpoint correctly identified all isolates with reduced susceptibility to benzylpenicillin. Although a small number of isolates were not identified by the 9-mm screening threshold used to detect reduced susceptibility to ampicillin, cefotaxime, or meropenem, none were resistant to these agents according to the reference method. Categorisation errors were confined to non-β-lactam agents, with total error rates of 4.7% and

3.1% after 6 and 8 h, respectively. Most VMEs were associated with the norfloxacin screening breakpoint, although only one isolate exhibited confirmed fluoroquinolone resistance. Additional VMEs were attributable to undetected inducible clindamycin resistance and discrepancies between disk diffusion and MIC categorisation for rifampicin.

Repeated testing of 73 pneumococcal isolates on an alternative agar medium yielded comparable zone diameter distributions and similar categorisation error patterns. Although readability after 6 h was higher on the alternative medium, this difference was considered more likely attributable to increased experience with early zone interpretation than to the agar medium itself. The main performance characteristics and key findings of Study II are summarised in Table 3.

Table 3. Summary of readability, categorical agreement and key findings in Study II

Species	Readability		Categorical agreement		Key findings and main challenges
	6 h	8 h	6 h	8 h	
Haemophilus influenzae (n=73)	19%	89%	–	98%	Ampicillin and cefotaxime resistance correctly identified; 8 h required for reliable reading
Streptococcus pneumoniae (n=112)	63%	98%	95%	97%	β-lactam screening successful; norfloxacin screening, rifampicin and inducible clindamycin resistance

To reduce the risk of false-susceptible results, ATUs were proposed for all species-antimicrobial-incubation time combinations. Application of the modified breakpoints reduced overall error rates to ≤0.9%, albeit at the expense of reportability, which decreased to 80% for *H. influenzae* and to 49% and 91% for *S. pneumoniae* after 6 and 8 h, respectively (Table 4).

Table 4. Impact of introducing ATUs on reportability and residual error rates in Study II

Species	Reportable results	Residual error rate
Haemophilus influenzae *(n=73)*	80%	≤0.4%
Streptococcus pneumoniae *(n=112)*	49% (6 h) 91% (8 h)	≤0.9%

Taken together, the findings from Studies I and II demonstrated that rapid phenotypic AST methods can be implemented in routine CMLs and provide reliable susceptibility categorisation within a single working day when interpreted using species-specific breakpoints and decision rules.

Opportunities to optimise the blood culture diagnostic pathway

- ✓ Considerable variation existed between Nordic clinical microbiology laboratories (CMLs) regarding blood culture logistics, incubation practices, service availability, and workflow organisation.
- ✓ Major reductions in blood culture turnaround times (TATs) were achieved through decentralised incubation and direct species identification from positive blood culture bottles.

In Study III, responses to the questionnaire on blood culture diagnostic practices were obtained from 49 of 76 Nordic CMLs (64%). According to the national coordinators, the responding laboratories showed no apparent systematic bias regarding hospital size, geographical location, or population coverage within their respective countries.

Decentralised blood culture incubation outside the CML was provided by 59% of laboratories, most commonly through incubators located in clinical chemistry departments or emergency departments. Continuous (24/7) blood culture incubation was available in 82% of CMLs; however, neither service was necessarily available at all

hospitals within the respective laboratory service areas. Processing of positive blood cultures remained constrained by laboratory opening hours. Only one laboratory provided continuous 24/7 microbiology service, but full work-up of positive blood cultures was not available throughout the night. Weekend services were typically more limited, with shorter opening hours, reduced staffing, and two laboratories closed on Sundays.

Microbiological processing at decentralised incubation sites was available at only a minority of laboratories and was limited to Gram staining. Rapid species identification and direct susceptibility testing from positive blood culture bottles were each routinely performed by 39% of laboratories, primarily using MALDI-TOF MS and modified disk diffusion methods. Only 19% of laboratories routinely performed both rapid species identification and direct susceptibility testing.

In approximately 60% of CMLs, the first preliminary report was communicated within 1 h of initiating positive blood culture processing and before 09:00. Comprehensive therapeutic advice linked to blood culture findings was routinely provided by 23% of laboratories, while direct communication of all or selected blood culture findings to an infectious diseases specialist was reported by 37%.

Although survey questions regarding quality indicators, including diagnostic TATs, were insufficiently answered for detailed analysis, delays exceeding 4 h from sampling to incubation were reported by 41% of the 22 laboratories providing such data.

Compared with a corresponding Swedish survey conducted in 2013, the availability of rapid species identification from short-incubation subcultures had increased significantly. Decentralised incubation and continuous blood culture incubation were also more common, whereas laboratory opening hours and the use of rapid species identification and susceptibility testing directly from positive blood culture bottles remained largely unchanged.

In Study IV, blood culture TATs were compared across a pre-intervention period and two consecutive post-intervention periods comprising 5,258, 9,785, and 10,499 positive blood culture bottles,

respectively. The interventions included decentralisation of blood culture incubation to all hospitals in the region with continuous (24/7) access to incubators and expanded transport services, followed by implementation of direct MALDI-TOF MS identification from positive blood culture bottles.

The first intervention (decentralised incubation) reduced the median time from blood culture collection to Gram stain reporting by 12.5 h compared with the pre-intervention period. The reduction was driven primarily by shorter times from sampling to incubation, with a smaller additional contribution from reduced delays between blood culture positivity and laboratory processing. The relocation of blood culture incubators also shifted the timing of blood culture positivity from predominantly late afternoon and evening hours to late night and early morning hours.

The second intervention (direct MALDI-TOF MS identification) reduced the median time to species identification for four common bloodstream pathogens by 14-18 h compared with the pre-intervention period. While reductions in time to incubation accounted for a substantial part of this improvement, implementation of direct identification from positive blood culture bottles further shortened time to species identification.

Taken together, Studies III and IV demonstrated that considerable opportunities to optimise blood culture diagnostics exist throughout the diagnostic pathway, extending beyond analytical methods alone.

Clinical impact of reduced diagnostic turnaround times

- ✓ Workflow interventions across the blood culture diagnostic pathway were associated with a substantial reduction in time to adequate antimicrobial therapy.
- ✓ Additional reductions in time to adequate therapy may be achieved through optimisation of post-analytical processes.

In Study IV, time to adequate antimicrobial therapy was evaluated in episodes of enterococcal and *Pseudomonas* bloodstream infections. Alongside the workflow interventions described above, reporting routines were modified to allow earlier communication of Gram stain and species identification results by permitting trained laboratory staff to issue preliminary reports directly.

Inadequate empirical therapy remained common throughout the study period, affecting approximately two-thirds of enterococcal bloodstream infections and between 43% and 63% of *Pseudomonas* bloodstream infections. In these episodes, optimisation of the blood culture diagnostic pathway was associated with a nearly 22-h reduction in median time to adequate antimicrobial therapy (Figure 6). The largest reduction in post-analytical delay was observed between Gram stain reporting and initiation of adequate therapy, as the median interval decreased from approximately 11 h to 5 h. In contrast, the interval between species identification reporting and adequate therapy decreased by approximately 2.5 h.

No corresponding reductions were observed in descriptive analyses of 30-day mortality, 90-day mortality, or length of hospital stay during the intervention periods.

Taken together, Study IV demonstrated that reductions in diagnostic turnaround times (TATs) translated into substantially earlier initiation of adequate antimicrobial therapy when empirical therapy was initially inadequate. Delays between microbiological reporting and treatment modification were reduced, although additional opportunities for optimisation remained beyond the analytical phase.

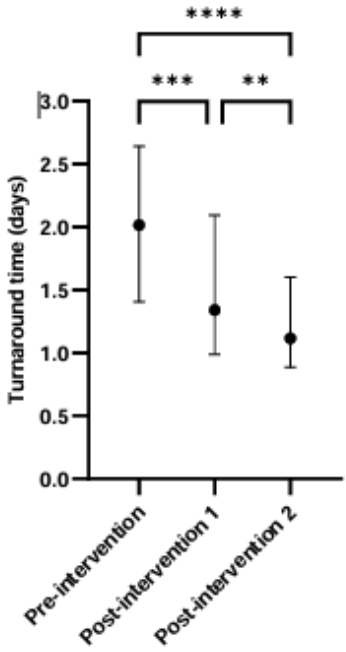


Figure 6. Time from blood culture collection to adequate antimicrobial therapy across study periods in episodes of bacteraemia caused by *Enterococcus* spp. and *Pseudomonas* spp. with inadequate empirical therapy.
Data are presented as median (IQR). Study periods are shown on the x-axis. Turnaround time is expressed in days (1.0 = 24 hours). Kruskal–Wallis with Dunn's post hoc test (**p < 0.01, ***p < 0.001, ****p < 0.0001)

Discussion

This thesis investigated several aspects of blood culture diagnostics, including rapid susceptibility testing, laboratory workflows, and the clinical impact of diagnostic optimisation. The methodological considerations and implications of the findings are discussed below.

Methodological considerations

Study designs and methodological approaches

To address the overall aims of the thesis, several complementary methodological approaches were employed.

Studies I and II were laboratory-based diagnostic evaluation studies. These studies evaluated the diagnostic accuracy, reproducibility, and robustness of rapid phenotypic antimicrobial susceptibility test (AST) approaches for clinically relevant bloodstream infection pathogens while maintaining a low risk of susceptibility misclassification.

In Study III, a survey was conducted among Nordic clinical microbiology laboratories (CMLs) to characterise contemporary blood culture diagnostic services and workflows. The survey design enabled the collection of comparable information from a large number of laboratories across countries with similar healthcare systems but varying organisational structures, while also encompassing laboratories operating under diverse geographical and logistical conditions. By mapping current practices, the study provided insights into factors that may facilitate or hinder timely reporting of bloodstream infection findings and identified potential bottlenecks as well as opportunities for optimisation throughout the diagnostic pathway.

Finally, Study IV evaluated the clinical impact of interventions targeting key bottlenecks identified along the blood culture diagnostic pathway. A quasi-experimental before-and-after design was employed to assess whether workflow and diagnostic interventions designed to shorten diagnostic turnaround times (TATs) translated

into earlier initiation of adequate antimicrobial therapy. This design enabled the interventions to be evaluated within routine clinical practice and provided insight into their impact under real-world conditions.

A major strength of Study I was the inclusion of prospectively collected clinical isolates from routine diagnostic practice, providing a realistic assessment of method performance under everyday laboratory conditions. However, as the study was based on routine clinical isolates rather than a deliberately constructed challenge collection, rare resistance mechanisms and particularly difficult-to-categorise isolates may have been underrepresented. Nevertheless, such isolates were included in the earlier method development study that formed the basis for the applied methodology and interpretive criteria (94). The multicentre design enabled evaluation of inter-laboratory reproducibility and ensured that between-laboratory variability was incorporated into the establishment of final clinical rapid AST (RAST) breakpoints. Inclusion of laboratories from southern Europe further increased the likelihood of capturing resistance phenotypes that would have been less common in a solely northern European study population, although it may also have introduced additional methodological variability due to differences in prior experience with disk diffusion testing.

In contrast, Study II used a deliberately selected isolate collection designed to challenge the method by including isolates spanning the susceptibility spectrum, with particular emphasis on resistant isolates and those with MICs close to clinical breakpoints. Such a design is particularly valuable during method evaluation because it increases the representation of isolates for which categorical interpretation is inherently difficult (136). Although this may result in higher observed error rates than would be expected in routine clinical practice, it provides a more stringent assessment of method performance and facilitates identification of susceptibility ranges where additional interpretive safeguards, such as areas of technical uncertainty (ATU), may be warranted. However, the single-centre design precluded formal assessment of inter-laboratory reproducibility. Nevertheless,

limited supplementary testing suggested that the method was robust to minor variations in testing conditions, and the findings are consistent with previous studies demonstrating similar methodological principles in other bacterial species (84-86).

Study III used a survey design that enabled collection of standardised and comparable information from a large number of geographically dispersed laboratories across the Nordic countries, an approach that would have been difficult to replicate through direct observation. However, survey findings depend on participant engagement and the accuracy of self-reported information. Although participation was not complete, laboratories from all Nordic countries were represented, and the national coordinators identified no apparent systematic bias regarding hospital size, geographical location, or population coverage among responding laboratories. Nevertheless, some degree of reporting bias and variation in interpretation of survey questions cannot be excluded.

Study IV evaluated workflow interventions that had already been implemented in routine clinical practice. Consequently, randomisation was not feasible, and a retrospective quasi-experimental before-and-after design was used to assess their impact. This design was well suited for evaluating the cumulative impact of workflow changes under real-world conditions and for assessing organisational interventions at the healthcare system level. However, as with all before-and-after studies, the influence of concurrent changes occurring during the study period cannot be completely excluded (137).

Internal validity and sources of bias

Internal validity concerns the credibility of observed study findings and the extent to which they are free from systematic error and bias (138). Several factors beyond the design-specific considerations discussed above may have influenced the interpretation of the findings.

The internal validity of Studies I and II was strengthened by the use of established reference methods. Reference testing was performed at the EUCAST Development Laboratory and, for most MIC determinations in Study II, at JMI Laboratories (USA), an internationally recognised laboratory with extensive experience in antimicrobial susceptibility testing.

In Study II, RAST results were evaluated against broth microdilution-derived MIC values, helping to ensure that susceptibility categorisation remained aligned with the MIC distributions underlying clinical breakpoint determination.

In Study I, EUCAST standard disk diffusion served as the primary reference method, as the methodology and preliminary RAST breakpoints had already been established in the preceding MIC-based method development study (94). Broth microdilution was nevertheless performed on a representative subset of isolates to assess the impact of using disk diffusion as the primary reference method. Similar error rates obtained with the two reference methods suggest that the influence on the overall findings was limited (Supplementary Table S4) (95). Although somewhat higher error rates were observed for *P. aeruginosa* when compared with broth microdilution, these were driven by a small number of discrepant categorisations near clinical breakpoints.

The potential for measurement error was considered in both laboratory studies. In Study II, inhibition zones were measured independently by two observers, reducing the influence of individual reading variability. In Study I, differences in experience with disk diffusion methodology may have contributed to some of the observed variability, as routine susceptibility testing practices differ between participating laboratories and the extent to which disk diffusion is used varies across Europe. This interpretation is supported by the observation that a small number of laboratories accounted for a disproportionate share of categorical errors, despite no clear association with resistance rates, resistance mechanisms, or out-of-range quality control results.

An additional source of potential measurement bias in Study II relates to quality control performance. For three antimicrobial agents

tested against *S. pneumoniae*, quality-control zone diameters were consistently lower than expected, and a corresponding downward shift was observed in the associated wild-type (WT) distributions. This concordance suggests a minor systematic influence on zone diameter measurements. However, the impact on the overall findings appeared limited, as the observed relationships remained consistent with previous methodological studies and major error rates were not increased.

Finally, the risk of selection bias due to exclusions was considered low. In Study I, only a small number of isolates were excluded because predefined study criteria were not fulfilled, most commonly due to incorrect species identification, making any associated selection bias likely negligible.

The internal validity of Study III depended on how well the questionnaire captured the intended study objectives and how respondents interpreted the questions. As in all questionnaire-based research, social desirability bias cannot be excluded and may have led respondents to overestimate adherence to recommended practices or report expected rather than actual performance (139). Since the survey focused on current laboratory practices, the risk of substantial recall bias was likely limited.

The questionnaire was developed within the framework of the Swedish Reference Group for Antibiotics (SRGA) by specialists in clinical microbiology and infectious diseases, helping to ensure that the questions were relevant to the study objectives and reflected current clinical practice. It was pilot-tested by three clinical microbiologists from different laboratories and revised based on their feedback before distribution, likely improving question clarity and interpretability. However, no formal validation of the questionnaire was performed.

To enhance the accuracy of the information collected, the questionnaire was directed to the physician responsible for blood culture diagnostics at each laboratory, or another staff member with detailed knowledge of local procedures. Distribution was coordinated by national coordinators with extensive experience in microbiological

diagnostics, who also assessed the representativeness of participating laboratories. Respondents could provide free-text comments to clarify their answers, and these were reviewed by two investigators to promote consistent interpretation and categorisation.

The response rate was 64%, which is generally considered acceptable for questionnaire-based surveys, although some risk of non-response bias remained (139). While no major imbalances were identified among responding laboratories, as assessed by the national coordinators, participating laboratories may nevertheless have differed systematically from non-participants. Laboratories with a particular interest in blood culture diagnostics may have been more likely to respond, potentially leading to some overrepresentation of highly engaged laboratories.

Variation in the interpretation of individual questions cannot be excluded. Differences in local terminology, organisational structures, and diagnostic practices between countries may have influenced how some questions were understood, and it is unlikely that all national differences in practice were fully captured despite extensive experience in blood culture diagnostics and the established Nordic collaboration among those involved in questionnaire development.

During data compilation, it became apparent that some questions, particularly those regarding the organisation and coverage of satellite incubators, might have benefitted from more detailed response options or subdivision into separate questions to better capture local laboratory routines and practices.

Questions regarding quality indicators and diagnostic TATs were often left unanswered, and reported TATs were frequently based on estimates rather than routinely collected statistics. Consequently, the only quality indicator for which sufficient data were available for analysis was the time from sampling to incubation, and these findings should therefore be interpreted with some caution. The limited availability of quality indicator data may itself be considered a study finding, highlighting barriers to quality monitoring and extraction of performance data from laboratory information systems and healthcare IT infrastructure.

The observed association between laboratory size and opening hours may partly have been influenced by differences in the distribution of responding laboratories across countries. Because the number of laboratories was limited, particularly within some countries, stratified analyses were not considered sufficiently robust. Consequently, residual confounding related to national differences cannot be excluded.

The internal validity of Study IV was influenced by the accuracy of recorded timestamps and treatment data, the consistency of data extraction and review procedures, and the before-and-after study design. Because interventions were introduced sequentially over time, their effects could not be completely separated from concurrent changes in laboratory workflows, staff experience, patient characteristics, sepsis management, and other secular trends. However, the magnitude of the observed effects and their close temporal association with the stepwise introduction of workflow changes targeting identified diagnostic bottlenecks suggest that the interventions were major contributors to the improvements observed.

Since historical laboratory information system data were only available for a limited period before the first intervention (decentralised incubation), the pre-intervention period was shorter than the two post-intervention periods, resulting in approximately half as many positive blood cultures as in the subsequent periods. This may have reduced the statistical precision of some species-level and patient-level analyses, particularly for subgroup analyses. However, the distributions and interquartile ranges were generally comparable across study periods, suggesting that the smaller sample size did not substantially increase variability in the observed data.

Data extraction and review were predominantly performed by a single investigator, ensuring consistent application of predefined criteria throughout the study. The absence of independent review may have introduced a risk of systematic bias in variables requiring subjective assessment. However, the primary study outcomes were based predominantly on objectively recorded timestamps and were therefore less susceptible to investigator-dependent bias. Some

variables, including determination of the first clinically available microbiological report, required manual review and interpretation, introducing a minor risk of classification error. Certain timestamps, including sampling time and time to antimicrobial administration, relied on routine clinical documentation. Minor discrepancies between actual and recorded times are therefore likely to have occurred, although such inaccuracies are unlikely to have differed systematically between study periods. Missing timestamps were uncommon and generally comparable across study periods, making a substantial impact on the observed results unlikely.

Assessment of treatment adequacy was based primarily on the intrinsic susceptibility profile of the identified species rather than isolate-specific susceptibility results. For *Enterococcus faecium*, β-lactam therapy was considered adequate only when susceptibility was documented, improving the accuracy of treatment classification. Although predefined criteria were applied consistently throughout the study, some uncertainty in treatment classification cannot be excluded, and any resulting inaccuracies would primarily be expected to affect analyses involving treatment adequacy and associated outcomes rather than the objectively recorded diagnostic TATs themselves. Nevertheless, the risk of substantial misclassification was considered low, given the use of predefined and consistently applied criteria.

Episodes managed at specialised intensive care units (ICUs) were excluded because prolonged admissions and complex underlying conditions may substantially influence time-to-treatment analyses. In these patients, multiple concurrent factors often influence treatment decisions, making the impact of individual microbiological findings more difficult to assess. Inclusion of these episodes might therefore have increased heterogeneity and reduced the ability to evaluate the effects of diagnostic workflow changes.

Analyses of treatment adequacy in relation to clinical outcomes were descriptive and unadjusted, and potential confounding cannot therefore be excluded.

External validity and generalisability

The external validity of the findings depends on the representativeness of the studied populations and settings, and on the similarity of healthcare systems, laboratory organisation, and clinical practice to those of other regions and institutions (138). For Studies I and II, important determinants of generalisability were the strain collections and laboratory settings included.

Study I was conducted as a multicentre validation study involving laboratories from different parts of Europe, capturing both inter-laboratory variation and diverse resistance epidemiology. Inclusion of laboratories from Southern Europe enabled evaluation in settings with higher resistance rates, while participation of Northern European laboratories contributed extensive experience in routine disk diffusion methodology. The close agreement between zone diameter distributions observed during method development and multicentre validation, together with the absence of systematic differences related to laboratory, blood culture system, agar manufacturer, or antimicrobial disk manufacturer, supports the robustness and broad applicability of the method. The external validity of the method is further strengthened by subsequent independent evaluations reporting similar performance across different laboratory environments (98-104).

Study II used a selected strain collection that was not intended to represent routine clinical isolates. This limits direct generalisability to everyday laboratory practice but was necessary to challenge the method under controlled conditions and evaluate its performance across a broad range of susceptibility patterns. Testing on two agar media increased the relevance of the findings across laboratories using different routine methodologies and, together with duplicate readings, increased the number of evaluable results and strengthened the robustness of the analyses.

While addressing different stages of method development and validation, Studies I and II provided complementary evidence regarding the performance of rapid disk diffusion-based susceptibility testing. Together with previous studies, the findings support the

feasibility of implementing the method in clinical microbiology laboratories. Because the approach builds on a well-established technique and requires no costly additional equipment, implementation may be feasible in a broad range of laboratory settings, although successful adoption remains dependent on local training and experience in zone interpretation and breakpoint-based categorisation.

The external validity of Study III was influenced by the representativeness of participating laboratories and the extent to which laboratory organisation and clinical practice resembled those in other settings. A major strength was the multicentre design, encompassing laboratories of different sizes, geographical locations, organisational structures, and healthcare systems across the Nordic region. Although the Nordic countries share many characteristics, including publicly funded healthcare systems, relatively low levels of antimicrobial resistance, and comparable laboratory standards, variation in laboratory organisation, diagnostic pathways, and service availability allowed assessment of blood culture diagnostics across a range of healthcare settings.

The questionnaire was directed to professionals with detailed knowledge of local blood culture diagnostic practices, supporting the relevance and reliability of the collected information. National coordinators also assessed the representativeness of participating laboratories, supporting generalisability within the Nordic region. However, the relative similarities between the participating countries may limit direct extrapolation of the findings to regions with substantially different healthcare systems, laboratory organisation, resource availability, or antimicrobial resistance patterns. While the primary aim was to describe blood culture diagnostic pathways within a Nordic context, many of the identified organisational challenges and diagnostic bottlenecks are likely to be relevant beyond the Nordic region, even if specific practices and implementation strategies differ between healthcare systems.

Study IV was conducted within a single regional healthcare system, limiting direct generalisability to other healthcare systems and laboratory organisations. Logistical factors such as geographical distances, laboratory opening hours, communication pathways, and information systems vary considerably between institutions and countries and may therefore influence the impact of workflow interventions. Consequently, the magnitude of the observed effects may not be directly transferable to all settings. Nevertheless, the evaluated interventions addressed common bottlenecks in the blood culture diagnostic pathway and were based on technologies and organisational approaches feasible in many contemporary clinical microbiology laboratories, although their implementation depends on local resources, infrastructure, and organisational structures (48, 49, 140). The specific interventions may differ between settings, but the principle of identifying and addressing major diagnostic bottlenecks is likely to be broadly applicable.

The study population comprised patients from a university hospital and regional hospitals, and may therefore be considered reasonably representative of patients with bloodstream infections in similar healthcare settings. Exclusion of patients managed in highly specialised intensive care units reduced the influence of exceptionally complex clinical pathways and may improve transferability to more general hospital populations. Episodes in which patients died, were transferred, or had treatment limitations established before microbiological results became available were also excluded, as the impact of diagnostic reporting on antimicrobial management could not be meaningfully assessed in these situations. Although these exclusions may have reduced representation of the most severely ill patients, their influence on the primary outcomes was likely limited.

Species-level analyses of time to adequate antimicrobial therapy were restricted to *Enterococcus* spp. and *Pseudomonas* spp. Although not representative of the broader population of patients with bloodstream infections, these pathogens commonly require microbiological confirmation to optimise antimicrobial therapy (25, 131, 132). The findings are therefore most applicable to clinical

scenarios where treatment modification depends on microbiological results.

General discussion

From rapid AST to microbiologistics

Historically, efforts to improve bloodstream infection diagnostics focused primarily on analytical performance within the microbiology laboratory. Early advances enabled pathogen identification within hours of blood culture positivity through the introduction of rapid identification methods, followed by approaches designed to shorten the time to AST results (81, 141-145). As rapid diagnostic technologies became increasingly available, attention gradually expanded beyond individual laboratory assays to encompass the entire diagnostic pathway. Delays in specimen transport, incubation, result reporting and clinical response were recognised as important determinants of the time to effective antimicrobial therapy (48, 49, 106, 140, 146). This broader perspective on the movement of samples, diagnostic information and clinical actions throughout the diagnostic process has been referred to as microbiologistics (43). The studies included in this thesis reflect this development, encompassing both methodological improvements in antimicrobial susceptibility testing and efforts to optimise the broader blood culture diagnostic pathway.

Studies I and II reflect early efforts to shorten TATs while maintaining reliable AST. Study I validated a standardised rapid disk diffusion method, whereas Study II showed that early susceptibility reading may complement rapid testing directly from positive blood cultures. The methods proved robust overall, but interpretation near the susceptibility breakpoint remained challenging.

Early reading inevitably entails a risk of incomplete separation of the zone diameter distributions of wild-type (WT) and non-wild-type (non-WT) populations (94). This phenomenon has been elegantly illustrated by Hombach and colleagues (Figure 7), who demonstrated that zone diameters continue to evolve during incubation and that

patterns of zone development vary both between and within species-antimicrobial combinations (85, 86).

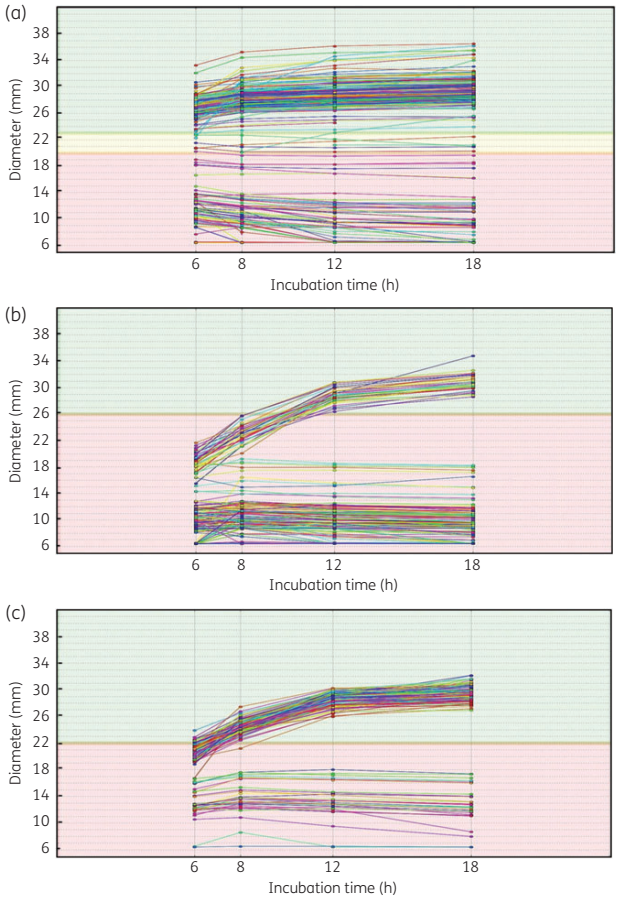


Figure 7. Diameter changes over time for selected drug/species combinations. Changes of the inhibition zone diameters over time read after 6, 8, 12 and 18 h of incubation: (a) ceftriaxone and *E. coli*; (b) penicillin G and *S. aureus*; and (c) cefoxitin and *S. aureus*. Each line represents an individual clinical isolate. The green area indicates susceptible categorisation according to EUCAST 2016 CBPs, the yellow area indicates intermediate categorisation, and the red area reflects resistant categorisation.

Reproduced from Hombach M, Jetter M. Fully automated disc diffusion for rapid antibiotic susceptibility test results: a proof-of-principle study. Journal of Antimicrobial Chemotherapy. 2017;72(6):1657-1662. Reproduced with permission from Oxford University Press.

The observed heterogeneity suggests that zone development is influenced by factors beyond the species-antimicrobial combination, including underlying resistance mechanisms that are unknown at the time of testing and therefore cannot be incorporated into routine interpretation (83). Together, these findings highlight the need for both species-specific and time-dependent breakpoints, as well as areas of technical uncertainty (ATUs) between susceptible and resistant categories. This was also evident in both Studies I and II and forms a fundamental prerequisite for reliable interpretation of rapid disk diffusion results. The limited separation between WT and non-WT populations after short incubation is likely explained by ongoing antibiotic diffusion and bacterial growth, as well as incomplete phenotypic expression of antimicrobial activity and resistance mechanisms during the early stages of incubation, reflecting differences in lag phase duration, generation time, and antimicrobial mode of action (76, 147). A thin layer of growth close to the disk may be visible at early reading time points even in WT isolates, reflecting incomplete cell lysis (94, 148). Conversely, heteroresistance and complex resistance phenotypes may obscure the early detection of resistance, particularly when resistant subpopulations are initially present at low frequencies (76, 149). Together, these phenomena may result in both false-susceptible and false-resistant classifications.

An additional source of categorisation uncertainty relates to isolates with AST results close to the clinical breakpoint, as even minor methodological or biological variation may shift the result across the breakpoint (85, 136, 150, 151). Such isolates are inherently challenging to categorise, even with standard susceptibility testing. Although training and experience are important for accurate zone interpretation (148, 152), some degree of categorisation uncertainty is likely unavoidable, and similar limitations should therefore be expected when applying RAST methods.

To facilitate interpretation after only a few hours of incubation, the EUCAST RAST methodology uses the inoculum obtained directly from positive blood culture bottles, resulting in substantially higher bacterial densities than in standard disk diffusion testing (97). Although this improves early readability, it introduces an additional

methodological difference that may influence zone diameter distributions (94, 97, 153).

While methodological challenges associated with RAST have gradually been addressed through standardised approaches, it became increasingly apparent that analytical improvements alone were insufficient to ensure earlier effective treatment. Similar observations were made in two large European surveys performed shortly before and after Study III (48, 140). Compared with laboratories in the rest of Europe, Nordic laboratories generally reported greater access to blood culture incubators, including more frequent use of decentralised incubators (60% vs 15%) and more extensive out-of-hours availability (82% vs 42%). Nevertheless, delays before incubation remained common, indicating that pre-analytical bottlenecks persisted despite these infrastructural advantages. Likewise, continuous handling and reporting of positive blood cultures remained uncommon in both the Nordic countries (0%) and the rest of Europe (<8%) (48, 140). Together, these findings suggest that access to diagnostic technologies alone is insufficient if organisational barriers elsewhere in the diagnostic pathway remain unresolved.

Rapid identification and susceptibility testing methods were increasingly available across Europe, with MALDI-TOF MS from short-incubation colonies and disk diffusion-based rapid susceptibility testing representing the most commonly adopted approaches. Direct identification from positive blood culture broth by MALDI-TOF MS was reported more frequently in Nordic laboratories than elsewhere in Europe (39% vs 29%). Laboratory service levels were consistently reduced during evenings, nights, weekends and holidays, and some laboratories reported no processing of positive blood cultures on Sundays (<10%) (48, 140). This is likely to limit the clinical impact of otherwise rapid diagnostic pathways, as access to laboratory services, antimicrobial stewardship support, and clinical decision-making outside routine working hours remains essential for timely action (118, 120).

Translating microbiologistics into clinical impact

Although rapid identification and susceptibility testing may provide clinically actionable results earlier, their impact on patient management depends on the performance of the entire diagnostic pathway and the timeliness of subsequent clinical action.

The clinical utility of blood cultures is highly dependent on preanalytical quality. Inadequate blood volumes reduce the probability of recovering microorganisms, particularly when bacterial concentrations are low (12, 110). Blood cultures obtained after initiation of antimicrobial therapy likewise have a reduced diagnostic yield, even in bottles containing antibiotic-adsorbing resins (42, 154, 155). Delayed incubation may also compromise microorganism recovery and increase the risk of false-negative results (113, 156), as growth detection in contemporary blood culture systems depends on ongoing microbial metabolism (157). Contamination can complicate result interpretation, as differentiating contaminants from true pathogens may be difficult. Such findings may lead to unnecessary broad-spectrum antimicrobial therapy, additional investigations and sampling, delayed diagnosis, prolonged hospitalisation, healthcare-associated infections, and increased healthcare costs (12, 158).

Several studies have evaluated the impact of providing 24/7 access to blood culture incubators, either through continuous on-site incubation or by placing incubators at affiliated hospitals or other peripheral healthcare facilities. Despite differences in organisational setup, these studies have consistently reported substantial reductions in diagnostic TATs, although the magnitude of the effect depends on the surrounding laboratory workflow and logistics (115, 156, 159, 160). Earlier adjustment of antimicrobial therapy has also been demonstrated, but without a corresponding reduction in hospital length of stay or mortality (159). Jensen et al. further highlighted the importance of prompt incubation by demonstrating that immediate loading may paradoxically prolong incubation-to-detection time while still shortening the clinically more relevant interval from blood collection to detection through elimination of pre-incubation delays

(156). These findings support the rationale for around-the-clock incubation, as a substantial proportion of blood culture bottles are collected outside routine laboratory working hours (159-161)

Beyond reducing turnaround times, decentralised 24/7 incubation shifted blood culture positivity towards the early morning hours, as demonstrated by Schaumburg et al. (161) (Figure 8). A similar pattern was observed in Study IV (Figure 9), allowing a greater proportion of positive bottles to be processed at the start of the working day.

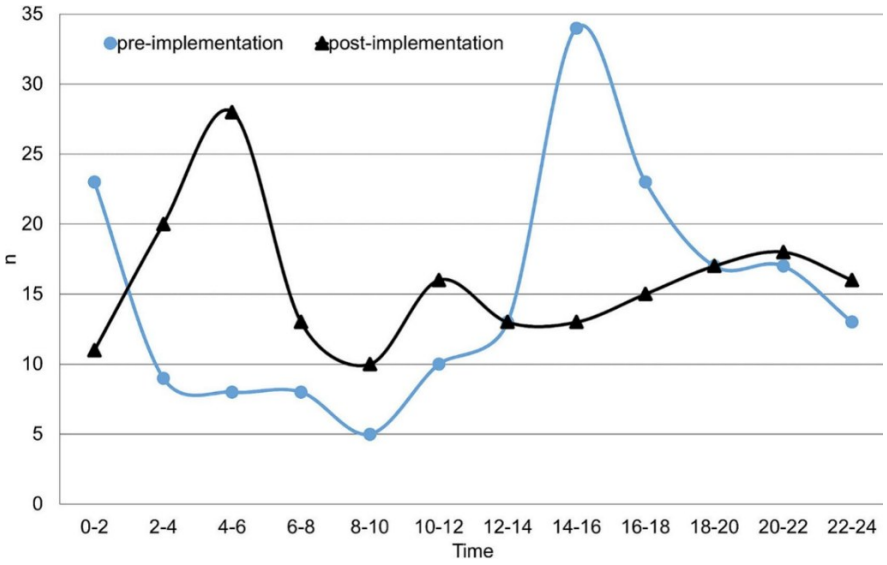


Figure 8. Distribution of blood culture positivity throughout the 24-hour cycle before and after implementation of decentralised 24/7 blood culture incubation. Reproduced from Schaumburg et al. (2024), licensed under CC BY 4.0.

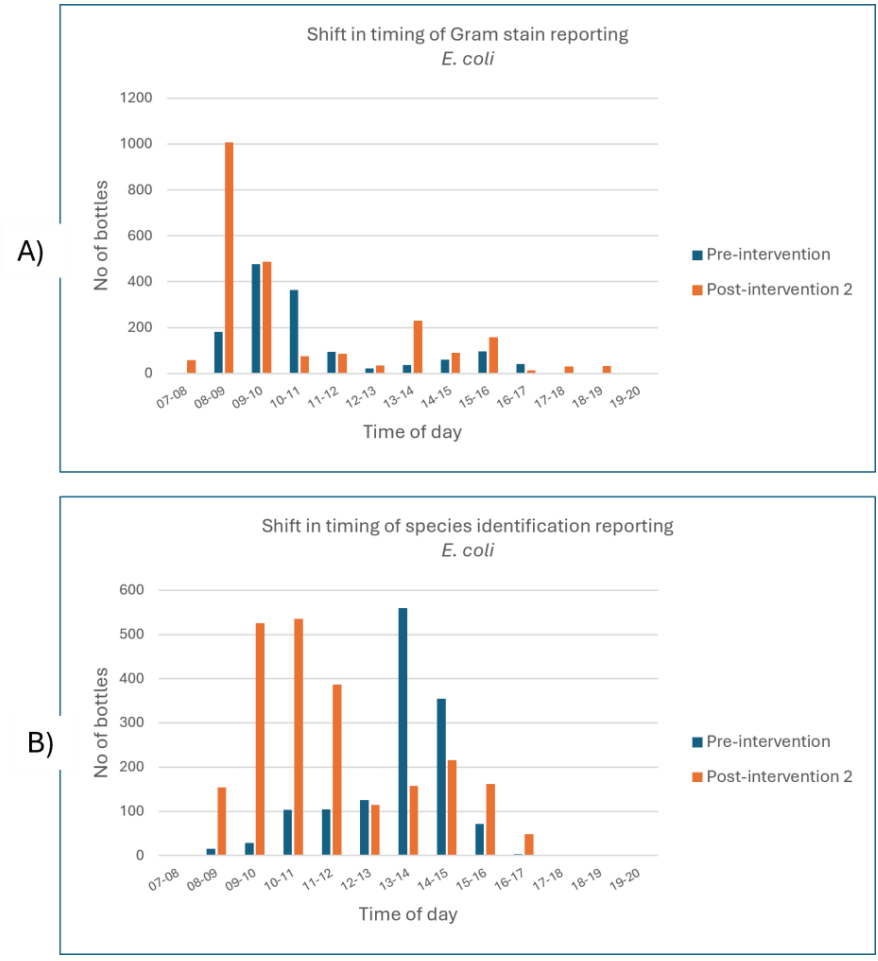


Figure 9. Shift in reporting times for *Escherichia coli* between the pre-intervention period and post-intervention period 2, following implementation of decentralised 24/7 blood culture incubation and direct species identification from positive blood culture bottles.
A) Distribution of Gram stain reporting times.
B) Distribution of species identification reporting times.

Consequently, clinically actionable information may become available before routine clinical decision-making takes place, increasing the opportunity for treatment adjustments and further diagnostic evaluation on the same day. This may partly explain why the reduction in post-analytical TAT in Study IV was more pronounced

for Gram-stain reporting than for species identification. While both results became available earlier, species identification may in some cases still have been reported after the main clinical decision-making window of the day.

Earlier positivity does not automatically translate into earlier clinical action. Realisation of the full benefit depends on the availability of laboratory and stewardship services outside routine working hours, and reduced weekend or out-of-hours service provision may therefore attenuate the impact of otherwise rapid diagnostic pathways. Numerous studies have evaluated the impact of rapid diagnostic methods on both diagnostic TATs and clinical outcomes. Although implementation of rapid pathogen identification methods consistently shortens reporting times, evidence for improvements in clinical outcomes is more heterogeneous. Studies have reported associations with shorter hospital stays, reduced healthcare costs, fewer healthcare-associated infections and lower mortality, but these benefits are most consistently observed when rapid diagnostics are combined with active antimicrobial stewardship programmes (123-125, 162).

In contrast, evidence that rapid AST (RAST) alone improves clinical outcomes remains limited. A recent Cochrane review found no reduction in mortality or length of stay but included only six heterogeneous randomised controlled trials comprising 1638 patients, most of which evaluated molecular resistance detection rather than contemporary phenotypic AST platforms (163). Nevertheless, RAST was associated with shorter times to appropriate therapy and earlier optimisation of antimicrobial treatment.

Similarly, the FAST trial, a multicentre randomised controlled trial evaluating modern phenotypic RAST in high-resistance settings within an established antimicrobial stewardship framework, demonstrated earlier treatment optimisation and more stewardship-guided interventions, without improving mortality, overall length of stay, or the primary composite clinical outcome (164). Notably, nearly two-thirds of participants were already receiving effective antimicrobial therapy at enrolment, leaving limited scope for further

benefit from RAST. In addition, access to optimal antimicrobial agents varied across study sites, limiting opportunities for treatment optimisation according to contemporary standards.

These findings are further supported by Halperin et al., who reported a 24-hour reduction in time to treatment modification and adherence to stewardship recommendations approaching 90% following integration of RAST into an established antimicrobial stewardship programme. Nevertheless, no significant reductions in mortality, hospital length of stay, or duration of antimicrobial therapy were observed (165).

Collectively, these observations suggest that RAST primarily facilitates antimicrobial optimisation, whereas translation into improved patient outcomes depends on factors beyond susceptibility testing alone, including patient selection, antimicrobial availability, stewardship activities, and the wider organisation of care.

Although randomised studies of RAST alone have shown limited effects on patient-centred outcomes, two recent observational implementation studies suggest that broader optimisation of the blood culture pathway may yield additional clinical benefits through earlier incubation, rapid identification, accelerated AST, and streamlined reporting (166, 167). Such multimodal interventions were associated with earlier optimisation of antimicrobial therapy, reduced use of broad-spectrum antibiotics, lower healthcare costs and, in some settings, shorter hospital stays and reduced mortality. Although the observed mortality benefits should be interpreted cautiously, as they were derived from observational before-and-after studies and may have been influenced by residual confounding, the findings suggest that clinically meaningful benefits may emerge when multiple sources of diagnostic delay are addressed simultaneously. Together, these studies support the concept that cumulative improvements across several consecutive steps in the diagnostic pathway may be required before measurable patient benefits become apparent.

As diagnostic pathways become increasingly complex, successful implementation of workflow improvements does not preclude the emergence of new bottlenecks elsewhere in the system. Reliable quality indicators and integrated information systems may facilitate identification of workflow bottlenecks, support targeted quality improvement initiatives, and help ensure that achieved improvements are sustained over time (168).

Why does rapid AST not consistently improve outcomes?

Several additional factors may contribute to the difficulty of demonstrating patient-centred benefits of RAST in clinical studies.
For RAST to influence patient management, susceptibility results must be sufficiently comprehensive and reliable to support confident escalation and de-escalation of antimicrobial therapy. The proportion of patients in whom RAST can meaningfully influence management is often restricted by effective empirical therapy, particularly in settings with low levels of antimicrobial resistance (25). Limited access to optimal antimicrobial agents may represent an additional barrier, especially in settings with a high prevalence of multidrug-resistant organisms (164). Furthermore, the impact of RAST is likely to vary according to pathogen, infectious syndrome, underlying comorbidity, and clinical severity (25, 131, 132).

While earlier susceptibility information may be particularly valuable in patients receiving inappropriate empirical therapy or in infections caused by multidrug-resistant organisms (24, 25, 164), its effect may be less pronounced when source control can readily be achieved or when outcomes are primarily determined by host factors rather than antimicrobial therapy (28, 131). Consequently, broad endpoints such as all-cause mortality and total hospital length of stay may underestimate the clinical value of RAST, whereas process measures and infection-related outcomes may represent more sensitive indicators of benefit (123, 162).

Finally, susceptibility results generated by currently available RAST methods may still become available at a stage when

opportunities to influence outcome have already diminished, despite being reported earlier than with conventional methods (23, 26). Furthermore, by the time susceptibility results become available, treatment decisions may already have been guided by empirical therapy, Gram stain findings, species identification, clinical response, source control measures, and other diagnostic investigations, thereby limiting the incremental value of the AST result itself.

Future perspectives

In the near future, efforts should focus on improving efficiency across the entire diagnostic pathway, from blood culture collection to timely optimisation of antimicrobial therapy. Emerging solutions, including drone- or robot-assisted specimen transport, automated logistics systems, decentralised diagnostic networks, and strategically distributed laboratory resources, may help reduce delays between specimen collection and laboratory processing, particularly in geographically remote settings (106, 169). The cost-effectiveness of these measures should be evaluated in relation to patient outcomes, healthcare utilisation, and antimicrobial use, as demonstration of downstream clinical and economic benefits may be important for wider implementation (166, 170).

By improving and standardising diagnostic workflows, turnaround times may not only become shorter but also more consistent. As diagnostic timelines become increasingly predictable, they may themselves provide clinically useful information. Understanding when clinically relevant microbiological results can reasonably be expected may help avoid unnecessarily broad-spectrum empirical antimicrobial therapy while awaiting susceptibility data. In clinically stable patients, persistently negative blood cultures beyond the expected time frame for pathogen detection may provide additional support for re-evaluating the need for continued empirical antimicrobial therapy (109).

However, generating diagnostic information more rapidly is only beneficial if it is effectively incorporated into clinical decision-

making. Improved integration of rapid diagnostics with hospital information systems, clinical decision-support tools, and antimicrobial stewardship programmes may help ensure that diagnostic results are communicated, interpreted, and translated into timely clinical action (119, 171).

Further gains in turnaround times and laboratory efficiency may also arise from increasing automation of susceptibility testing. Automated zone reading, continuous image-based analysis, and AI-assisted interpretation may facilitate around-the-clock reporting of susceptibility results and enhance the clinical utility of early-reading approaches based on standard disk diffusion methodologies (76). Together with improved understanding of expected diagnostic timelines, these developments may support more informed and individualised antimicrobial decision-making (76, 106, 109). Further research is also needed to determine which antimicrobial stewardship interventions most effectively limit the emergence and spread of antimicrobial resistance (172), including the contribution of rapid diagnostic technologies to antimicrobial use and resistance development. Although categorical S/I/R interpretations remain essential for clinical decision-making, future approaches may increasingly integrate quantitative susceptibility data, pharmacokinetic/pharmacodynamic considerations, host factors, and infection characteristics to allow more individualised antimicrobial therapy (173).

Achieving faster, more informative, and increasingly individualised antimicrobial therapy will depend on continued advances in RAST technologies. Current RAST methods remain limited by trade-offs between speed, diagnostic coverage, cost, and operational complexity (174). Future AST platforms will need to address these limitations while maintaining analytical robustness and clinical applicability to achieve widespread clinical implementation. For culture-independent testing, reliable performance at very low bacterial concentrations will represent an additional challenge (174, 175).

A wide range of technologies are currently being explored to address these challenges, although most remain at the proof-of-

concept or early clinical evaluation stage. Selected emerging approaches are summarised in Table 5 and include molecular, phenotypic, omics-based, and single-cell platforms, each offering distinct advantages and limitations. Although these technologies share the goal of accelerating susceptibility testing, they rely on fundamentally different biological principles and provide different types of information.

Molecular diagnostic approaches enable direct detection of resistance determinants from clinical samples without requiring extensive bacterial growth. However, resistance genes do not always translate into clinically relevant susceptibility predictions, particularly when resistance is influenced by gene regulation, complex genetic interactions, or yet undiscovered mechanisms. Metagenomic approaches remain challenged by low bacterial biomass and difficulties determining whether detected resistance determinants originate from the causative pathogen or from co-detected colonising organisms or contaminants (76, 78).

Phenotypic methods directly measure expressed antimicrobial susceptibility, irrespective of the underlying resistance mechanism. However, their performance is constrained by biological processes such as lag phase, antimicrobial uptake, and bacterial growth dynamics (76, 147). Consequently, many next-generation AST platforms aim to reduce dependence on growth-based measurements and instead assess metabolism, cell morphology, membrane properties, division dynamics, nanomotion, and single-cell responses to antimicrobial exposure (76, 93, 174).

Metabolomic approaches attempt to circumvent the limitations imposed by lag phase by measuring metabolic activity that persists even while bacterial cells adapt to a new environment. This allows antimicrobial effects to be detected earlier than with conventional growth-based methods. However, although metabolic alterations may precede measurable growth differences, sufficient time is still required for discriminatory responses to develop following antimicrobial exposure, with reported response times ranging from minutes to a few hours depending on the biomarker, bacterial species, antibiotic, and analytical platform (147, 174).

Transcriptomic and proteomic approaches bridge the gap between genotype and phenotype by assessing gene expression and protein production, thereby identifying biological responses that may not be predictable from genomic data alone. However, interpretation of these data remains challenging owing to dynamic expression patterns and uncertain translation into clinically actionable susceptibility information (76, 78).

To overcome some of the limitations of population-based approaches while reducing the dependence on large bacterial inocula, single-cell approaches have attracted considerable interest. These methods measure changes in biomass, growth dynamics, or metabolic activity at the level of individual cells, while also enabling the detection of resistant subpopulations and heteroresistance (76, 93, 174, 175).

Owing to the limitations of individual technologies, there is growing consensus that the earlier susceptibility testing is performed, the less it can rely on measurable growth and the more it must depend on physiological, metabolic, or transcriptional responses. Consequently, future AST strategies will likely combine multiple complementary approaches and increasingly incorporate artificial intelligence and machine-learning methods for data integration, interpretation, and antimicrobial resistance prediction (78, 174-177).

Table 5. Emerging AST technologies: strengths and limitations.

Category	Technology	Biomass requirement	Strength	Limitation
Phenotypic	Conventional growth-based AST	High	Measures phenotypic susceptibility	Slow, requires growth
	Single-cell AST (microscopy/ cytometry)	Low	Detects heteroresistance, persisters, and rare resistant subpopulations	Challenging in polymicrobial samples
	Raman-spectroscopy-based AST	Low	Measures single-cell metabolic and physiological responses	Sensitive to cell-to-cell variation
Molecular	Whole-genome sequencing (WGS)	High	Identifies resistance genes, mutations, and mechanisms	Does not assess expression or functionality
	Metagenomics	Very high	Detects multiple pathogens and resistance determinants directly from specimens	Difficult to assign resistance genes to specific species; affected by host DNA
Omics	Transcriptomics	High	Measures transcriptional response to antibiotic exposure	Expression may not predict protein function or phenotype
	Proteomics	High	Detects expressed resistance proteins	Does not capture all resistance mechanisms or protein activity
	Metabolomics	Moderate to high	Measures functional response to antibiotics	Population-based approaches may miss heteroresistance and rare resistant subpopulations

Note. Artificial intelligence and machine learning may support data integration, resistance prediction, and clinical interpretation across molecular, phenotypic, and omics-based AST technologies rather than constituting distinct AST platforms.

Further reductions in diagnostic TAT will likely require direct-from-blood testing that bypasses blood culture incubation, which currently represents the largest single contributor to overall diagnostic TAT. However, low bacterial concentrations in blood often necessitate enrichment, concentration, or nucleic acid amplification prior to analysis (76, 174). Emerging technologies, including microfluidics, single-cell AST, and molecular diagnostics approaches, have demonstrated proof-of-concept feasibility for generating susceptibility results directly from whole blood within a few hours. If successfully translated into routine clinical practice, such technologies may provide actionable results before administration of the second antibiotic dose (174, 178).

From an antimicrobial stewardship perspective, diagnostic approaches capable of distinguishing bacterial from viral infections are of particular interest for reducing unnecessary antibiotic use. Several host-response assays are already commercially available and combine multiple biomarkers of the host immune response, ranging from simple point-of-care tests to advanced machine learning-based algorithms. Early studies have demonstrated promising diagnostic performance, particularly in acute respiratory infections, with potential to reduce inappropriate antibiotic prescribing (179).

Current host-response assays largely rely on a limited number of protein biomarkers. However, ongoing developments aim to capture the broader complexity of the immune response. By integrating multiple layers of information, including protein biomarkers, leukocyte gene-expression profiles, cellular phenotypes, and other biological signatures, future approaches may further improve the ability to determine not only whether a patient has an infection, but also whether it is caused by bacteria, viruses, fungi, or other pathogens.

Beyond etiologic diagnosis, host-response profiling may facilitate earlier recognition of sepsis and identification of biologically distinct sepsis endotypes, potentially enabling more individualised prognostication and supportive or immunomodulatory treatment strategies. Ultimately, future diagnostic platforms may integrate

pathogen identification and host-response profiling within a single assay, providing simultaneous information on both the causative microorganism and the host response to infection (180).

Taken together, future improvements in bloodstream infection diagnostics will depend not only on novel diagnostic technologies but also on continued optimisation of diagnostic workflows and effective integration of diagnostic information into clinical decision-making. Advances in microbiologistics, antimicrobial stewardship, rapid AST, and host-response diagnostics are likely to be complementary rather than competing approaches towards more timely and increasingly individualised antimicrobial therapy.

Conclusion

This thesis demonstrates that microbiological diagnostics in bacteraemia can be substantially accelerated without compromising diagnostic quality. The findings further indicate that optimisation of the entire blood culture pathway, rather than individual diagnostic steps alone, is required to achieve meaningful reductions in time to adequate antimicrobial therapy. The main conclusions of this thesis are:

- ✓ Rapid antimicrobial susceptibility testing can provide reliable susceptibility results within 8 hours of blood culture positivity for the most common bloodstream infection pathogens, provided that species- and incubation time-specific interpretive criteria are applied.
- ✓ Rapid antimicrobial susceptibility testing approaches are feasible for implementation in routine clinical microbiology laboratories.
- ✓ Significant organisational and logistical bottlenecks remain throughout Nordic blood culture diagnostic pathways and contribute substantially to diagnostic delays.
- ✓ Decentralised blood culture incubation and rapid pathogen identification significantly shorten diagnostic turnaround times, with the greatest gains achieved through earlier incubation and continuous access to blood culture incubation.
- ✓ As diagnostic turnaround times decrease, delays related to subsequent laboratory handling and clinical response to microbiological results become increasingly important determinants of time to adequate antimicrobial therapy.
- ✓ Monitoring total and fractional turnaround times provides a useful framework for identifying bottlenecks, evaluating interventions, and supporting quality improvement in blood culture diagnostics.
- ✓ The greatest reductions in time to adequate antimicrobial therapy are achieved when organisational and diagnostic interventions are combined, highlighting the importance of optimising the

entire blood culture pathway rather than isolated components of the diagnostic workflow.

List of abbreviations

AMR	antimicrobial resistance
AST	antimicrobial susceptibility testing
ATU	area of technical uncertainty
BMD	broth microdilution
CA	categorical agreement
CML	clinical microbiology laboratory
ECOFF	epidemiological cut-off value
EDL	EUCAST Development Laboratory
ESBL	extended-spectrum beta-lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FISH	fluorescence in situ hybridisation
ICU	intensive care units
MALDI-TOF MS	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
mE	minor error
ME	major error
MIC	minimum inhibitory concentration
MH	Mueller-Hinton
MH-F	Mueller-Hinton Fastidious
MRSA	methicillin-resistant *S. aureus*
PCR	polymerase chain reaction
PD	pharmacodynamic
PK	pharmacokinetic
SOFA score	Sequential Organ Failure Assessment score
TTP	time to positivity
VME	very major error
Non-WT	non-wild-type
WT	wild-type

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