UTILITY AND IMPACT OF DETECTING CLINICALLY IMPORTANT BACTERIA WITH SMALL-SCALE AND AUTOMATED NUCLEIC-ACID AMPLIFICATION ASSAYS

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ACADEMIC DISSERTATION

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ABSTRACT

During the last decade, nucleic acid-based amplification techniques (NAATs) have revolutionized the way clinical microbiology laboratories diagnose human pathogens. Modern automated NAATs require considerably less hands-on time and testing is much simpler than with conventional detection methods. The combination of ease of performance and speed, has made real-time NAATs appealing alternatives to conventional culture-based or immunoassay-based testing methods for diagnosing various infectious diseases.

However, in this era of implementing new technologies, it is crucial to focus not only upon the possibilities but also upon the pitfalls of the technology. Failure to do so may increase the cost of implementation, and put the new technology at risk of losing reputation in the eyes of the clinicians. This thesis deals with the basic principles behind modern NAATs and the implementation and utility of some of these modern assays in clinical diagnostics.

The thesis consists of six studies on three new fully automated NAAT platforms, the BD Max, the GenomEra CDX, and the GenRead system. All platforms were used for the screening of toxigenic *Clostridium difficile* in faecal specimens in comparison with the routine laboratory methods. In addition, the GenomEra CDX system was used for the detection of *Staphylococcus aureus* and the marker of methicillin resistance from various sample matrixes, and the detection of *Streptococcus pneumoniae* from blood cultures.

All assays showed excellent sensitivity and specificity for the target microbes. Moreover, all platforms decreased the analysis time significantly as compared to conventional laboratory methods, although variation between the NAAT test systems were seen. As its best, the total turnaround-time was less than 30 minutes with the GenRead, 55 minutes with the GenomEra, and 90 minutes with the BD Max system. The platforms had different sample throughput capacity and space requirement, as well. The lightweight and battery-powered

GenRead instrument with isothermal NAAT based assay proved to be most flexible system for clinical diagnostics, enabling mobile analytics in both laboratory and near patient.

Molecular techniques such as real-time PCR and isothermal amplification combined with modern robotics can provide significant advantage in laboratory diagnostics for detection of pathogenic bacteria. In this study, the three NAAT platforms proved to be suitable for rapid testing of *C. difficile*, methicillin-susceptible and -resistant *S. aureus*, and *S. pneumoniae* from various sample types.

TIIVISTELMÄ

Viime vuosikymmenen aikana nukleiinihappopohjaiset monistustekniikat ovat mullistaneet kliinisesti merkittävien patogeenien mikrobiologista laboratoriodiagnostiikkaa. Modernit automatisoidut nukleiinihaponosoituslaitteistot vaativat huomattavasti vähemmän käsityöaikaa ja testaus on yksinkertaisempaa kuin tavanomaisilla laboratoriomenetelmillä. Helppokäyttöisyys ja nopeus on tehnyt reaaliaikaisista nukleiinihappotekniikoista houkuttelevia vaihtoehtoja perinteisille viljely- tai antigeenipohjaisille analyysimenetelmille erilaisten tartuntatautien diagnostiikkaan.

Uusien tekniikoiden ilmaantuessa on kuitenkin tärkeää keskittyä paitsi niiden tuomiin mahdollisuuksiin myös niissä piileviin haasteisiin. Mikäli näin ei tehdä, saattaa huolimaton käyttöönotto lisätä laboratorion kustannuksia ja asettaa uuden tekniikan vaaraan menettää arvoaan asiantuntijoiden silmissä. Tämä väitöskirjatyö käsittelee modernien nukleiinihaponosoitusmenetelmien perusperiaatteita sekä joidenkin näiden tekniikoiden hyödyntämistä kliinisen mikrobiologian diagnostiikassa.

Väitöskirjatyö koostuu kuudesta osatutkimuksesta, jotka käsittelevät kolmea automatisoitua nukleiinihaponosoituslaitteistoa; BD Max:ia, GenomEra CDX:ää ja GenRead:iä. Kaikkia kolmea laitetta käytettiin toksigeenisen *Clostridium difficilen* seulonnassa ulostenäytteistä ja saatuja tuloksia verrattiin kliinisten laboratorioiden käytössä oleviin rutiinimenetelmiin. Tämän lisäksi GenomEra CDX -laitteistoa hyödynnettiin *Staphylococcus aureuksen* ja metisilliiniresistenssigeenin havaitsemiseen erilaisista näytemateriaaleista sekä *Streptococcus pneumoniaen* havaitsemiseen veriviljelynäytteistä.

Kaikki testisysteemit osoittivat erinomaista herkkyyttä ja tarkkuutta kohdemikrobien suhteen. Lisäksi testatut laitteistot pienensivät analyysiaikaa merkittävästi tavanomaisiin laboratoriomenetelmiin verrattuna, joskin tässä testijärjestelmien välillä haivatiin selvää vaihtelua. Parhaimmillaan kokonaistes-

tiaika oli alle 30 minuuttia GenRead-laitteella, 55 minuuttia GenomEra-laitteella ja 90 minuuttia BD Max -laitteella. Myös laitteistojen näyteanalyysikapasiteetissä ja tilavaatimuksessa havaittiin eroavuuksia. Kevyt ja akkukäyttöinen GenRead-laite, jossa hyödynnettiin isotermistä nukleiinihaponosoitusmääritystä, osoittautui joustavimmaksi kliiniseen diagnostiikkaan mahdollistaen mukana kuljetettavan analyysiyksikön, joka soveltuu sekä laboratorioon että lähellä potilasta tehtävään analytiikkaan.

Molekyylitekniikat, kuten reaaliaikainen PCR ja isoterminen nukleiinihaponosoitus yhdistettynä nykyaikaiseen robotiikkaan, voivat mahdollistaa merkittävän diagnostisen hyödyn patogeenisten bakteerien analytiikkaan. Tässä väitöskirjatyössä tutkitut kolme nukleiinihappopohjaista testisysteemiä osoittautuivat erittäin soveltuviksi *C. difficilen*, metisilliiniherkkien ja -resistenttien *S. aureus* sekä *S. pneumoniae* -bakteerien nopeaan tunnistukseen useista eri näytetyypeistä.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. **Hirvonen JJ**, Nevalainen M, Tissari P, Salmenlinna S, Rantakokko-Jalava K, Kaukoranta S-S. Rapid confirmation of suspected methicillin-resistant *Staphylococcus aureus* colonies on chromogenic agars by a new commercial PCR assay, the GenomEra MRSA/SA Diagnose. *Eur J Clin Microbiol Infect Dis*. 2012, 31(8):1961–1968 doi: 10.1007/s10096-011-1527-0.

II. **Hirvonen JJ**, von Lode P, Nevalainen M, Rantakokko-Jalava K, Kaukoranta S-S. One-step sample preparation of positive blood cultures for the direct detection of methicillin-sensitive and -resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci within one hour using the automated GenomEra CDX™ PCR system. *Eur J Clin Microbiol Infect Dis*. 2012, 31(11):2935–2942. doi: 10.1007/s10096-012-1644-4.

III. **Hirvonen JJ**, Mentula S, Kaukoranta S-S. Evaluation of a New Automated Homogeneous PCR Assay, GenomEra *C. difficile*, for Rapid Detection of Toxigenic *Clostridium difficile* in Fecal Specimens. *J Clin Microbiol*. 2013, 51(9):2908–2912. doi: 10.1128./JCM.01083.-13.

IV. **Hirvonen JJ**, Kaukoranta S-S. Comparison of BD Max Cdiff and GenomEra C. difficile molecular assays for detection of toxigenic *Clostridium difficile* from stools in conventional sample containers and in FecalSwabs. *Eur J Clin Microbiol Infect Dis*. 2015, 34(5):1005-9. doi: 10.1007/s10096-015-2320-2.

V. **Hirvonen JJ,** Seiskari T, Harju I, Rantakokko-Jalava K, Vuento R, Aittoniemi J. Use of an automated PCR assay, the GenomEra S. pneumoniae, for rapid detection of *Streptococcus pneumoniae* in blood cultures. *Infect Dis (Lond)*. 2015, 47(11):796-800. doi: 10.3109/23744235.2015.1063157.

VI. **Hirvonen JJ**, Matero P, Siebert C, Kauppila J, Vuento R, Tuokko H, Boisset S. Novel portable platform for molecular detection of toxigenic *Clostridium difficile* in faeces: a diagnostic accuracy study. *Eur J Clin Microbiol Infect Dis*. 2017, 36(5):783-789. doi: 10.1007/s10096-016-2860-0

ABBREVIATIONS

ASP	antibiotic stewardship program
BORSA	borderline oxacillin-resistant *Staphylococcus aureus*
BSI	blood-stream infection
Ca	Calsium
CDI	*Clostridium difficile* infection
cDNA	complementary DNA
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CoNS	coagulase-negative staphylococci
C_T	cycle threshold
dNTP	deoxyribonucleotide triphosphate
DNA	deoxyribonucleic-acid
DNAemia	DNA circulating in blood
DNase	deoxyribonuclease
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
Eu	Eurobium
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EXPAR	exponential amplification reaction
FRET	fluorescent resonance energy transfer
GDH	glutamate dehydrogenase
HAI	hospital-acquired infection
HCAI	healthcare-associated infection
HDA	helicase-dependent amplification
IA	immunoassay
IC	internal control
iNAAT	isothermal NAAT
LAMP	loop mediated isothermal amplification
LBM	liquid-based medium
LED	light-emitting diode
Li	Lithium
LoD	limit of detection
MALDI-TOF MS	matrix-assisted laser deionization-time of flight mass spectrometry
mecA	methicillin-resistance mediating gene A (coding for PBP2')
mecC	methicillin-resistance mediating gene C (coding for PBP2c)
MIC	minimal inhibitory consentration

MOD-SA	moderately resistant *Staphylococcus aureus*
MRCoNS	methicillin-resistant coagulase-negative staphylococci
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-susceptible *Staphylococcus aureus*
NAAT	nucleic acid amplification technique
NASBA	nucleic acid sequence-based amplification
NCBI	National Centre for Biotechnology Information
NEAR	nicking enzyme amplification reaction
NEMA	nicking enzyme mediated amplification
NGS	next-generation sequencing
NPV	negative predictive value
nuc	*Staphylococcus aureus* nuclease gene
orfX	open reading frame X
OS-MRSA	oxacillin-susceptible-MRSA
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
POC	point-of-care
PPV	positive predictive value
RCA	rolling circle amplification
RNA	ribonucleic-acid
RNase	ribonuclease
rRNA	ribosomal RNA
RT	reverse transcriptase
RT-PCR	reverse transcriptase-PCR
SA	*Staphylococcus aureus*
SCC*mec*	staphylococcal cassette chromosome *mec*
SDA	strand displacement amplification
SIBA	strand invasion based amplification
SMART	signal-mediated amplification of RNA technology
spa	*Staphylococcus aureus* protein A gene
SPC	sample-processing control
ssDNA	single-stranded DNA
SSSR	self-sustained sequence replication
qPCR	quantitative PCR
TAT	turnaround time
Tb	Terbium
TcdA / *tcdA*	*Clostridium difficile* toxin A / gene
TcdB / *tcdB*	*Clostridium difficile* toxin B / gene
TMA	transcription-mediated amplification
URS	unitized reagent strips
VCH	Vaasa Central Hospital

1 INTRODUCTION

Microbial populations are extremely ubiquitous in the environment. Through everyday activities such as breathing, eating, and touching, the human body is constantly exposed to these microorganisms. Although most of these organisms are benign and can live on humans (on our skin and mucus membranes) as part of the normal microbiota, some organisms can act as pathogens and cause damage to the host. The physician, following clinical examination of a patient, may suspect the presence of certain infectious agents and order microbiological tests from various patient samples, such as infected tissues, pus, blood, faeces, urine, or sputum. Even though pathogenic microbes represent a minority in the whole microbial population, they cause substantial morbidity and mortality worldwide (1-10). Thus, proper diagnostics is essential for rapid identification of infectious agents and timely treatment of a patient.

In clinical (diagnostic) microbiology laboratory, the main role is to screen specimens for pathogens typically associated with given disease or infection. Until the 21st century, non-nucleic-acid based techniques such as direct microscopy, culture, and immunological tests were the main techniques for the detection of infectious agents in the clinical specimen (11). Although these conventional techniques form the basis of diagnostic microbiology and, *e.g.*, the culture is still considered as the "gold standard" for detection of many pathogenic bacteria, it is well known that they possess limitations (12,13). The most important limitation of culture is that it is a time consuming and labour-intensive method. Moreover, culturing requires viable organism to be present in the specimen, which sets certain requirements for transport and storage conditions of the specimen, and slowly growing bacteria, fastidious or uncultivable bacteria may remain undetectable (14-18).

To improve the detection of clinically important bacteria, significant technological advancements have been made in recent years. During the first decade of 21st century, various nucleic acid amplification-based techniques (NAATs)

have become available for clinical microbiology laboratories to diagnose infectious diseases (13,19-22). Technological advances in, *i.e.*, real-time PCR techniques, nucleic acid sequencing, and DNA microarrays have invigorated the field and created new opportunities for laboratories to support patient care. Simultaneously, the dependency of microbiology laboratories on time consuming culture based techniques has been reduced because of NAATs.

2 REVIEW OF LITERATURE

NAATs are based on three basic steps; nucleic acid extraction, target amplification, and target detection and each step has its own critical elements that should be understood when using these techniques.

2.1 Techniques for nucleic acid extraction and purification

The preanalytical phase aims to release microbial nucleic acids present in the specimen. This step is critical for the whole process (23). When deciding which method to use, the type of pathogen sought is to be considered. Due to the differences in cell wall structures, some bacteria such as gram-negative enterobacteria can be lysed easily, whereas gram-positive organisms such as staphylococci and spore forming clostridia can be very difficult to lyse.

Nucleic acid extractions can be categorized roughly into either chemical or physical techniques. In chemical extraction, detergents and proteolytic enzyme, such as proteinase K are used to solubilize the bacterial cell wall and cytoplasmic membranes to release the nucleic acid (24,25). Physical extraction, on the other hand, consists of, *e.g.*, boiling of the specimen or using ultra sound sonication. The crude sample lysate formed from either of the techniques contains both DNA and RNA. Since bacterial RNAs are highly unstable, with an average half-life of about 3 minutes for fast-growing bacteria, extracted samples need to be stabilized if RNAs are to be analysed instead of DNA (26,27). In order to isolate RNA from DNA, DNase with chaotropic agent guanidium thiocyanate and detergent can be used to digest DNA, denature proteins, and inhibit ribonucleases (28). By contrast, if lysate containing DNA only is required, RNase can be added, instead of DNase, to enhance the degradation of RNA (26).

Although the crude sample lysate can be used directly in many amplification assays, the presence of large amounts of cellular and other materials in clinical

specimens, *e.g*. proteins, carbohydrates, urea, bile salts, and nitrates, in complex mixtures often impedes the subsequent nucleic acid amplification reactions (Table 1) (29-39). Thus, these components are considered PCR inhibitors and they should be removed prior, during, or after extraction process or avoided during amplification and detection. In addition to the substance group, the concentration of the molecular compound is important for its inhibitory effect (32). Hence, widely applied approach for the removal of PCR inhibitors is the dilution of the sample or the extracted sample lysate (40,41). However, the dilution is accompanied by a decrease in assay sensitivity. Instead of dilution, PCR inhibitors may be removed effectively by sample treatment with activated carbon, using physical and chemical extraction techniques simultaneously or consecutively with or without filtration (Table 1) (32). Albeit non-specimen related inhibitory component, the polymeric surface of a labware, *e.g*. sample container or reaction vessel, may have a high adsorption of DNA decreasing the yield of nucleic acid and the sensitivity of the test (Table 1) (42,43). Thus, molecular grade labware with low adsorption of DNA should be used for NAATs.

Table 1. Examples of PCR inhibitors, their mechanisms of action, and general methods for removal of inhibitors

Affecting step	Inhibitor	Mechanism of action	Method for removal of inhibitor	Reference
Extraction	The polymeric surface of a labware	High adsorption of DNA, especially if high ionic strength sample buffer is used	Labware with low adsorption of DNA	(42,43)
	Nucleases	Degrade template DNA and RNA	Physical or chemical inactivation	(33)
Amplification	Metal ions (*e.g*. Ca^{2+})	Inhibition of polymerase via competitive metal ion binding	Removal of metal ions *e.g.* with chelating agents	(34-36, 44)
	EDTA[a]	Inhibition of polymerase via chelation of metal ions such as Mg^{2+}	Removal of EDTA by filtration or dialysis	(37)
	Proteases	Degrade polymerase	Physical or chemical inactivation	(37)
	Urea	Degrade polymerase	Sample dilution	(38)
Detection	Haemoglobin	Increase background fluorescence	Usage of label molecules resistant to background fluorescence	(39)
	Nucleases	Degrade probes	Physical or chemical inactivation	(37)

[a]EDTA = ethylenediaminetetraacetic acid

To simplify the extraction and nucleic acid purification processes, a number of commercial manufacturers have developed manual extraction kits for clinical laboratories (13). These kits differ according to the method, cost, and time required for extraction. This variability allows flexibility in choosing the kit that suits best to the needs of the laboratory. Commercialized extraction kits typically use noncorrosive agents making them safe to use by laboratory personnel (13). However, while these kits are generally inexpensive and easy to use, they have several drawbacks. Processing of samples requires multiple manipulations. As the number of samples to be extracted increases, augments the risk of contamination. Hence, manual extraction can be a laborious, time-consuming process which requires the undivided attention of the technologist in order to ensure optimal results.

To make it even simpler, some manufactures have developed commercially available extraction reagents suitable for automated platforms from a wide variety of clinical specimens (see also chapter 2.5). Most commonly used kits for automated systems consists of either a chemical extraction with nucleic acid purification by a spin column technology (filtration) (45,46) or a chemical and/or physical extraction with nucleic acid purification by a magnetic silica particles technology (47,48). According to Nickoloff, in the spin column technology, the lysate buffering conditions (salt and pH) are adjusted to allow optimal binding of the nucleic acid to the membrane before the sample is loaded onto the spin column (49). These conditions also ensure that protein and PCR inhibitors are not retained on the membrane. In the end, purified nucleic acid is eluted from the spin column in a concentrated form in either buffer or water (49). According to Tan *et al.*, nucleic acid purification and isolation using magnetic particles is based on the ultra-small and uniform magnetic nanoparticles, which are constructed with an iron oxide magnetic core and a silica layer, and coated with a functional group, such as carboxylic acid, thiol, or streptavidin, containing moieties (50). Again, nucleic acid is captured in an adjusted lysate buffer conditions (salt and polyalkylene glycol concentration), but now by the functional group, which acts as a bioaffinity absorbent. During washing steps, nucleic acid is separated from other molecules and PCR inhibitors. In the final

step, nucleic acid is eluted from the magnetic particles into a buffer solution or water (50).

To control the success of nucleic acid extraction and removal of inhibitors, an internal control (IC) is typically included into the extraction process. The IC should be either an artificial DNA target or a target from human (cellular) DNA which is amplified and detected along with the microbial nucleic acid target(s). If the sample yields a negative results and IC is not amplified then a re-extraction and/or sample purification is needed.

2.2 Target selection (primers and probes)

In order to detect anything from the extracted sample lysate, one should define the target(s) of interest. As the genome of bacteria (prokaryotes) differs from human cells (eukaryotes), it has been reasonably easy to design oligonucleotide primers and/or probes suitable for the NAATs used in clinical microbiology (51). Primers and probes are the main components of nucleic acid-based detection systems and have been the subject of multiple studies (52,53). Primers are specific oligonucleotide strands that flank the target nucleic acid sequence to be amplified and are complementary to opposite strands of the target. Primers are typically between 15 and 30 bases long and do not have to be exactly the same size. However, it is crucial that the melting temperatures of the two primer/template duplexes are identical within 1–2 °C, in order to ensure specific annealing to the target sequence (54). Probes, on the other hand, are for the specific detection of the target nucleic acid sequence. They are a fragment of DNA or RNA of variable length (usually 50-100 bases long) labelled with a molecular marker of either radioactive or (more recently) fluorescent molecules (13). Both, primers and probes are reviewed further in chapters 2.3 and 2.4.

The target primer sequence must be unique in order to identify a specific pathogenic organism or an organism group among normal microbial flora, (*e.g.*, *Staphylococcus aureus*, *Streptococcus pyogenes* [group A streptococci] or

Mycobacterium genus in wound swabs or sputum samples), to quantify microbes (*e.g.*, cytomegalovirus in plasma), or to identify unique virulence genes (*e.g.*, verotoxin genes or *Clostridium difficile* toxins A and B genes in faeces) or genes or mutations associated with antimicrobial resistance (*e.g.*, the mediator for methicillin resistance, the *mecA* gene in *S. aureus* or mutations in *rpoB* gene associated with rifampicin resistance in *M. tuberculosis*) which can occur across strains or species (13). For accurate species identification, the target nucleic acid sequence should be conserved and exist only in the target organism. Most commonly used targets for microbial identification are species-specific "housekeeping" genes or the species-specific region of ribosomal 16S gene (53,55). These targets genes demonstrate only minimal evolutional alteration as they are vital for the organism. Other target genes such as virulence or resistance genes, though, may vary due to evolution and move from a one group of microbes to another, which should be taken into account in target selection and in primer and probe design (56,57).

In order to avoid primer sequence cross-reactivity (*i.e.*, false-positive results) and to confirm assay specificity it is recommend to search for the intended primer sequence in a DNA database such as the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/) or perform a decent method verification with known microbes in complex specimen matrices such as stool (13).

2.3 Nucleic acid amplification

Nucleic acid amplification can be categorised basically into two main groups, signal amplification and target amplification. In signal amplification techniques, the concentration of target nucleic acid does not increase. The increased analytical sensitivity comes from increasing the concentration of labelled molecules attached to the target (58). As a result, the signal is directly proportional to the amount of target sequence present in the specimen. This reduces concerns about false-positive results due to cross contamination, as the number of target molecules is not altered. Moreover, signal amplification techniques are not affected by the presence of enzyme inhibitors in specimens,

as they are not dependent on enzymatic processes to amplify the target. An example of signal amplification technique is hybrid capture assays where the target DNA in the extracted sample is denatured and hybridized with a specific RNA probe (59). Then, the DNA-RNA hybrids are captured by anti-hybrid antibodies coated on the surface of a reaction tube. Alkaline phosphatase-conjugated anti-hybrid antibodies bind to the immobilized hybrids and the detection is achieved with a chemiluminescent substrate in a luminometer.

Despite the benefits of signal amplification techniques, target amplification is more commonly used in clinical laboratories because it provides greater analytical sensitivity and broader technical applications. Target amplification techniques are discussed more closely in the following chapters (2.3.1, 2.3.2, and 2.3.3.).

2.3.1 Polymerase chain reaction (PCR)

The development of the polymerase chain reaction (PCR) by Mullis and coworkers (60,61) was a milestone in biotechnology and heralded the beginning of molecular diagnostics. Although other strategies have also been developed and utilized in molecular microbiology, PCR is still the most widely used NAAT.

At its simplest, a PCR consists of (extracted) target DNA, oligonucleotide primers, a heat-stable DNA polymerase, a mixture of deoxyribonucleotide triphosphates (dNTPs; dATP, dCTP, dGTP, and dTTP), salts such as $MgCl_2$ and KCl, and a buffer. These are mixed together to create a reaction mixture. To initiate the PCR, the reaction mixture is heated to separate the two strands of target DNA and then cooled to permit the primers to anneal to the target DNA in a sequence-specific manner. The DNA polymerase initiates the extension of the primers at their 3' ends toward one another. The primer extension products are then dissociated from the target DNA by heating. (21) Each extension product, as well as the original target, can serve as a template for subsequent rounds of primer annealing and extension.

At the end of each cycle, the PCR products are theoretically doubled. The whole procedure is carried out in a programmable thermal cycler that controls precisely the temperature of each step, the lengths of time the reaction mixture is held at the different temperatures, and the number of cycles. Ideally, after 20 cycles of PCR a 10^6-fold and after 30 cycles a 10^9-fold amplification occurs. (21) In practise, however, the theoretical amplification is not achieved completely due to the presence of possible residual inhibitors and unoptimized reaction conditions.

2.3.2 Reverse transcriptase-PCR (RT-PCR)

As it was originally described, conventional PCR was suitable for DNA amplification only. In order to amplify RNA targets, though, some technical modifications for PCR were required. Thus, reverse transcriptase (RT)-PCR was developed. In this process, cDNA is first produced from RNA targets by reverse transcription and then the cDNA is amplified by PCR (21). The first RT-PCR used two enzymes, a heat-labile RT such as avian myeloblastosis virus RT and a thermostable DNA polymerase. Because of the temperature requirements of the heat-labile enzyme, cDNA synthesis had to occur at temperatures below the optimal annealing temperatures of the primers. This caused problems in terms of both nonspecific primer annealing and inefficient primer extension (21). These problems have been overcome by the development of a thermostable DNA polymerase derived from *Thermus thermophiles* that under the proper conditions (typically at a temperature between 45–80 ^{o}C) can function efficiently as both an RT and a DNA polymerase (62).

2.3.3 Isothermal amplification

Unlike PCR, isothermal nucleic acid-base amplification (iNAAT) is a technique where the target amplification process can be performed at a single temperature. This eliminates the need for expensive thermal cyclers. Furthermore, as the amplification reaction occurs constantly and not only during programmed cycles, the time to target detection can be as short as 15–30 minutes (63,64). Isothermal amplification can be divided into several methodological “sub-

groups" such as, nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (SSSR), transcription-mediated amplification (TMA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), exponential amplification reaction (EXPAR), nicking enzyme amplification reaction (NEAR), nicking enzyme mediated amplification (NEMA), rolling circle amplification (RCA), strand displacement amplification (SDA), and signal-mediated amplification of RNA technology (SMART) (65-74).

NASBA, SSSR, and TMA are very similar techniques, combining three different enzymes to specifically amplify RNA or single-stranded DNA (ssDNA) within a time range of up to 90 minutes (75). These enzymes are an avian myeloblastosis virus reverse transcriptase, RNase H, and a T7 RNA polymerase (66,67,75). Although the amplification occurs isothermally (at 41 ^{o}C), NASBA, SSSR, and TMA require an initial heating step of either 95 ^{o}C (for DNA) or 65 ^{o}C (for RNA) to prepare accessible single strands for the T7 RNA polymerase (75). The single-stranded products can be detected *e.g.*, by hybridization probes to create a homogeneous, kinetic amplification system similar to real-time PCR (see chapter 2.4.) (76).

In LAMP, a strand-displacing DNA polymerase is employed with four to eight specifically designed primers to recognize six to eight distinct regions on the target DNA at a constant temperature of 60 ^{o}C (75). According to Tröger *et al.* different assays based on LAMP have run-times approximately 60 minutes and have shown a high amplification efficiency (75). Moreover, during the amplification, the DNA polymerase produces a large amount of pyrophosphate ion by-product, which forms an insoluble precipitate with divalent metallic ions, that can be observed with a turbidimeter or simply with the naked eye (63,71,75). LAMP amplification products can be detected also by common post-amplification techniques for NAATs (see chapter 2.4.).

The HDA mimics the naturally occurring process of DNA replication (69), in which the double-stranded target DNA is unwinded by a helicase at a temperature of 37 °C. Additional proteins assist in the reaction by stimulating the helicase and preventing re-hybridization of the separated ssDNA targets (77), enabling primer annealing and extension by a DNA polymerase. Target detection can be achieved within 120 minutes (75). Recent developments with a heat-stable helicase from *Thermoanaerobacter tengcongensis* allows the reaction to occur at a temperature of 45 °C to 65 °C without the additional assisting proteins and with reduced assay run-time (30–60 minutes) (75).

In EXPAR and its recent improvements NEAR and NEMA, a strand-displacing DNA polymerase with short oligonucleotides (referred as triggers) are employed to initiate amplification at a nick created by a nicking enzyme (70). The reaction yields a high amount of DNA under isothermal conditions within minutes (70). According to Tröger *et al.*, the three techniques are most suitable for the detection of small microRNAs (75). The amplification products can be detected by a variety of standard methods such as real-time fluorescence, and capillary electrophoresis detection (75).

RCA exploits specific linear oligonucleotide probes (called padlock probes) containing two target specific sequences at each end (72,73). After hybridization, these probes circularize and ligate serving as template for the Phi29 bacteriophage polymerase, which continuously elongates the product displacing the generated strand. According to Tröger *et al.*, the dual recognition in combination with a ligation reaction ensures specificity of detection (75). Optimized RCA has shown to be able to detect extremely low copy numbers of genomic bacterial DNA in less than 65 minutes (78-80).

SDA includes two reaction steps, target generation and target amplification (68,81). During the first step, after the heat induced strand separation of the double-stranded (dsDNA), the first sequence specific primers introduce a restriction site into the product and bumper primers, which bind adjacent to the first primer, will be elongated by a strand-displacing DNA polymerase. Then,

a strand-limited restriction endonuclease or nicking enzyme will cleave the restriction sites and polymerase regenerates single stranded copy molecule with each displacement step (68,81). However, according to Tröger *et al.*, SDA has two major limitations as compared to other iNAATs, a long processing time (more than two hours) and the limited use of suitable restriction enzymes (75).

SMART is an iNAAT, which consist of two single stranded primers both capable of hybridizing abreast to the target sequence and to each other forming a three-way junction (74). According to Wharam *et al.* and Tröger *et al.*, after the junction formation, the shorter extension primer is elongated by a DNA polymerase, based on the sequence of the template primer, which includes a T7 RNA polymerase promotor sequence. Subsequently, the transcription template with promotor sequence will be produced, allowing for a multiplicative production of transcription RNAs by T7 RNA polymerase (74,75). SMART enables efficient target amplification and detection at 41 ^{o}C within an hour. However, the amplification of dsDNA still needs an initial denaturation step of over 90 °C.

2.4 Post-amplification detection and analysis

In order to visualize the amplified target(s) during or after amplification process, additional techniques such as gel analysis, colorimetric detection, line probe assays, hybridization arrays, sequencing, or enhanced thermal cycler platforms allowing real-time target detection, are needed.

The first detection method was visualization of amplified product in agarose gel after electrophoresis and ethidium bromide staining. This technique is, however, laborious and time-consuming, and it depends on a toxic dye (21). Moreover, gel electrophoresis requires separate post-PCR facilities to minimize the risk of nucleic acid cross contamination. Thus, gel analysis is no longer favoured in routine microbiological diagnostics. Additional alternatives, other than real-time detection platforms, are *e.g.*, colorimetric microtiter plate, allele-specific hybridization, hybridization arrays, and sequencing (21).

According to Nolte *et al.* colorimetric microtiter plate systems are more convenient alternatives to traditional gel and blotting techniques. The amplified product is captured in microtiter plate wells by specific oligonucleotide probes coating the plastic surface. Bound product is detected by a colour change that takes place after addition of an enzyme conjugate and the appropriate substrate (21). The redeeming feature of this technique is that it can be carried out in microtiter plate washers and readers commonly found in clinical laboratories.

In allele-specific or line probe assays, a series of probes with poly(T) tails are attached to nitrocellulose strips. Biotin-labelled PCR product is then hybridized to the immobilized probes on the strip. After hybridization, streptavidin labelled with alkaline phosphatase is added and binds to the biotinylated hybrids. The pattern of hybridization provides information about the nucleic acid sequence of the amplicon. This method is capable of detecting single-nucleotide polymorphism and is used, *e.g.*, for identification of mycobacteria, analysis of drug resistance mutations, and genotyping of some microbial species (82,83).

Hybridization arrays are small plates or chips in which hundreds or thousands of oligonucleotides are attached on their solid surface in precise patterns. A labelled amplification product is hybridized to the probes, and hybridization signals are mapped to various positions within the array (84,85). Hybridization arrays have a number of applications in microbiology, including microbial and host gene expression profiling and diagnostic sequencing (21).

Direct sequencing has been predicted to be the next revolutionizing technique in clinical microbiology diagnostics. Briefly, the nucleotide order of a given DNA or RNA is determined by laser and fluorescent dye terminator or pyrophosphate degradation (86-88). Although direct sequencing of PCR products

is a powerful research tool, its routine use in the clinical microbiology laboratory depends upon the development of high-throughput and cost-efficient platforms with integrated databases and data analysis software (21).

The disadvantage of distinct post-amplification protocol is, however, that it will add an additional phase to NAAT analysis as compared to real-time detection platforms. Thus, the latter approach has been more popular in clinical microbiology diagnostics.

2.4.1 Real-time PCR

The term real-time or quantitative PCR (qPCR) refers to methods in which the target amplification and detection steps occur simultaneously in the same tube (homogeneous). These methods require special thermal cyclers with precision optics that can monitor the fluorescence emission from the sample wells. Also, a computer software is needed to support the thermal cycler. The software monitors the data throughout the PCR at every cycle and generates an amplification plot for each reaction (kinetic PCR).(13) Similar instrumentation can be utilized in iNAATs, without the thermal cycling steps, though. The time required for target detection in real-time PCR depends on the time required for thermocycling, and the speed of thermocycling depends on how quickly the instrument can change the temperature (21). Some instruments, such as the MIC qPCR cycler (Bio Molecular Systems), which uses magnetic induction, can change the temperature at a rate of over 20 ^{o}C per second, permitting analysis of up to 48 samples in as little as 25 minutes.

During the early cycles of real-time amplification, there is commonly only little change in the fluorescence signal. This initial signal level defines the baseline for the amplification plot. An increase above the baseline indicates the detection and accumulation of amplification product. However, in some instances a fixed fluorescence threshold, which is set above the baseline, is needed. The cycle threshold (C_T) is defined as the cycle number at which the fluorescence passes the fixed threshold. The C_T improves the assay specificity, *e.g.*, if the

sample template contains high background fluorescence or if there is any risk for primer dimer formation.

In its simplest format, the real-time amplification product is detected as it is produced by using fluorescent dyes, *e.g*, SYBR Green I, that preferentially bind to double-stranded DNA (89-91). In the unbound state, the fluorescence is relatively low, but when the dye is bound to double-stranded DNA, the fluorescence is greatly enhanced. However, the dye will bind to both specific and non-specific PCR products. The specificity of the detection can be significantly improved by using fluorescent resonance energy transfer (FRET) probes, such as the TaqMan probes (Thermo Scientific), labelled with fluorescent dyes or with combinations of fluorescent and quencher dyes (13,92-94). FRET probes are dual-labelled, one fluorescent dye serving as a reporter, which emission spectrum is quenched by the second fluorescent dye. During target amplification, the nuclease degradation of the hybridization probe releases the reporter dye, resulting in an increase in the peak fluorescent emission (13,95). The intensity of fluorescence is related to the amount of the product. Since the signal is generated only when the primer and probe are bound to the same template strand, the assay specificity is increased.

Similar to the TaqMan probes, molecular beacons also make use of FRET detection with fluorescent probes attached to the 5' end and a quencher attached to the 3' end of an oligonucleotide substrate (96). In contrast to TaqMan probes, which are quite short oligonucleotide strands that are cleaved during amplification, molecular beacons are hairpin-shaped, medium length, probes that remain intact and rebind to a new target during each reaction cycle. Due to this, molecular beacons enable both real-time and end-point detection of the target molecule. When free in solution, the close proximity of the fluorescent probe and the quencher molecule prevents fluorescence through FRET. However, when molecular beacon probes hybridize to a target, the fluorescent dye and the quencher are separated resulting in the emittance of light upon excitation (96).

Scorpion probes are modified versions of molecular beacons, that combine an amplification primer with a molecular beacon (97,98). The hybridization kinetics of Scorpion probes are generally faster than those of molecular beacons because the primer and probe are located on the same molecule.

Probes containing fluorescent quencher may, however, be problematic, as they may yield background fluorescent signal during amplification process. With dark quencher probes, though, this issue can be avoided. These probes contain a fluorophore on the 5' end and a non-fluorescent quencher molecule on the 3' end (99). The fluorescence is quenched when the probe is a random coil and emitted when the probe anneals to the target sequence. Similar to molecular beacons and Scorpion probes, dark quencher probes are not degraded by the DNA polymerase. In addition, dark quencher probes incorporate a hybridization-stabilizing compound, known as a minor groove binder (99). This is a small, crescent-shaped molecule that is covalently linked to the 3' end of the probe that spans about 3 to 4 nucleotides and snugly fits into the minor groove of DNA, where it forms hydrogen bonds with the template. The minor groove binder allows for the use of shorter probes because of the increased T_m and enables improved T_m levelling, which increases the specificity of the detection reaction.

Since real-time NAATs incorporate homogeneous target amplification (primers) and detection (probes), the instrument programming becomes challenging as compared to conventional PCR. As an example, the annealing temperature for probes can be several degrees below the melting temperature of the primers. Moreover, primers and probes may have a high potential to form secondary structures, including self and cross-hybridization with other oligonucleotides in the reaction, which increases the risk of nonspecific amplification and decreases the assay specificity (13,95). This may become an issue, especially with complex assays such as isothermal amplification platforms, in which the reaction temperature remains constant and the molecules collide by chaotic nature.

2.4.2 Multiplex PCR

The combination of multiple primer sets into a single amplification reaction (multiplex assays) for simultaneous detection of several targets is becoming more popular in clinical diagnostics (100). Co-amplification and detection of many targets in a single tube provide a great advantage over monoplex assays, as it broadens the detection capacity of the particular assay. Multiplex assays can be very useful when testing specimens from patients presenting with non-specific symptoms attributable to a number of different pathogens. Multiplex assays, however, have proved to be even more complicated to develop and are usually less sensitive than real-time amplification assays with single primer sets (21). The primers and probes used in multiplexed reactions must be carefully selected in order to have similar annealing temperatures and lack complementarity.

Despite the complexity of designing multiplex assays, the introduction of platforms equipped with optics capable of excitation and detection of multiple fluorophores in a closed system in real-time amplification has made multiplex pathogen detection a simple and viable option for molecular diagnostics in routine clinical laboratories (12). Recently, larger multiplex panels have become available for use in clinical diagnostics (20,101). These test panels are typically capable of low-density multiplexing of four to six unique targets. The ability to multiplex only up to six targets can, however, be a limitation, especially when numerous microorganisms are able to cause similar symptoms such as gastroenteritis, upper respiratory illness, or bacterial sepsis (102,103). This limitation is imposed by the number of optical channels and inability to differentiate between fluorescent dyes with similar emission wavelengths. The optics on early PCR platforms, such as SmartCycler II (Cepheid) and first-generation BD Max (BD) were limited to a maximum of four channels. Newer platforms, including the GeneXpert (Cepheid), LightCycler 2.0 (Roche), second generation BD Max (BD), and ABI 7500 Fast Dx and ABI Quant-Studio (ABI) are capable of detection in up to six different channels (21).

2.5 NAAT automation and robotics

As described above, NAATs consist of three major steps: specimen processing (nucleic acid extraction and purification), nucleic acid amplification, and target detection. The most labour-intensive step is sample processing and it has represented the biggest challenge for manufactures of automated test systems (104,105). In the past several years, though, there have been considerable advances in this area with the availability of both semi-automated and fully-automated systems (12,23,26,48,105-108). The basic idea in all automated sample processing systems is that the manual steps, such as pipetting, and moving of the sample tubes, are replaced with robotic arm. Automation of the nucleic acid extraction process and robotics offers clinical laboratories several advantages, including ease of use, limited handling of the sample, reduced opportunity of cross contamination, improved reproducibility, and, for some systems, post-elution functions such as mixing samples and mastermix for amplification assays (105,108).

The currently available automated systems vary in the types of nucleic acid extraction methods they provide including total nucleic acid, DNA-only, and RNA-only protocols (21). Furthermore, some systems provide protocols for various specimen types and volumes, variable elution volumes, the availability of target-specific and/or generic target extraction methods, and specimen throughput (21). Automated systems range from high-throughput instruments, such as MagNa Pure (Roche) and *m*2000 specimen processor (Abbott), to those designed for a small number of specimens with random access capabilities, such as BioRobot EZ1 (Qiagen) (105,108,109). The extraction time is typically 1–3 hours, depending on the system used.

In addition to specimen processing, considerable advances in automation have also been made with the availability of real-time amplification and detection systems (chapters 2.4.1 and 2.4.2) , which enable rapid target amplification, higher capacity of samples per test run, variation in reaction volume, accurate optics, and detection of various fluorescent probe types (13,21,95).

Due to the recent technological developments, many microbiological laboratories have begun relying on centralized facilities with automated high-throughput instruments but at the same time, by simplification of the testing process and miniaturisation of testing platforms, NAAT based point-of-care (POC) diagnostics has become more popular (110-112). Careful consideration of facility requirements, personnel qualifications, and work flow design is essential before implementing NAATs in microbiology laboratories, like implementating any new type of testing method (113).

2.6 Implementation and utility of NAATs in clinical microbiology laboratory

The appearance of modern NAATs has made many microbiologists reconsider the ways of performing clinical microbiological diagnostics (15,114). In the field of clinical bacteriology, NAATs have long been considered supplements to classical routine analyses (15). In recent years, though, increasing number of NAAT assays have become substitutes for classical microbiology tests. Main advantages of NAATs include the ability to detect even low numbers of pathogens signifying increased sensitivity and the possibility to obtain quick results. For example, NAATs can detect fastidious and slowly propagating bacteria such as *M. tuberculosis* in a few hours (115-118), as well as bacteria, such as *Mycoplasma* sp., that are difficult to grow as visible colonies with current culture-based methods (119,120).

However, the powerful exponential amplification achieved by NAATs produce a risk for false-positive signals due to contamination. Since up to 10^{12} copies of a specific target sequence can be generated in a single amplification reaction, even minimal amounts of aerosol can contain thousands of DNA copies. If conventional PCR with post-PCR detection techniques such as gel electrophoresis is used, the essential measure to avoid cross contamination is to separate the pre-PCR and the post-PCR work areas – ideally in two separate buildings. However, in practise the "gold standard" (level 3 facilities) for a PCR laboratory performing conventional PCR and "in-house" assays should be considered

in clinical laboratories. According to Kwok, proper PCR laboratory includes four separate rooms, at minimum, with unidirectional workflow (from room 1 through 4) and unidirectional airflow if individual airflow cannot be installed (121). Each room should be separated from any of the other rooms, and, if possible, a positive air pressure in rooms 1 and 2 and a negative air pressure in room 4 should be obtained. Moreover, rooms 1 and 2 should have a laminar air flow bench. In room 1, no DNA is permitted. This room is used for production of mastermixes and setup of the individual PCR analysis except addition of sample DNA. Room 2 is used for extraction of clinical samples and adding the extracted nucleic acids to the premade PCR mixes. In Room 3, the thermal cyclers are placed. In Room 4, post-amplification procedures such as detection can be performed (121). In addition, all working surfaces should be cleaned before and after use, preferably with a reagent that destroys nucleic acid such as a 5% bleach solution. Gloves should be changed frequently, at least before beginning each of the separate tasks required in a dedicated work area and should always be changed if moving from one laboratory room to another. Moreover, the use of aerosol-resistant pipette tips and pipette tips long enough to prevent specimen contact with the pipetter aids in the prevention of specimen contamination (122). Conventional PCR applications have high requirement for space and skilled technologists and have, therefore, been considered to pose "a high methodological complexity". They are not practical for smaller laboratories and are mainly seen in larger and more centralized laboratories (13,54,113,121).

In contrast to conventional PCRs, real-time NAATs are performed in closed systems. Consequently, there is no need for individual air-controlled and post-PCR laboratory rooms. The risk for release of amplified nucleic acids into the environment, *i.e.*, contamination of subsequent analyses, is negligent compared with conventional methods (13,54,113). However, real-time NAAT platforms are able to provide sensitivities and specificities equivalent to conventional PCRs combined with hybridization analysis.

Platforms like the *m*2000 (Abbott), and Cobas AmpliPrep (Roche) feature a two-instrument three-step system whereby automated nucleic acid extraction is followed by automatic addition of all reagents required for a real-time PCR on one instrument and real-time amplification on another instrument (108,123). According to Marshall *et al.*, and Hochberger *et al.*, the main advantage of these platforms is that up to 96 specimens per run can be processed with minimal hands-on time. Thus, these systems make high-throughput molecular detection possible even in mid-size laboratories lacking space and highly skilled technologists. However, in these platforms prepared specimens must still be moved manually from the extractor to the thermocycler to complete analysis (108,123). In addition, proper cleaning of instrument components, processing blocks, and surfaces is necessary. The need for human intervention and a narrow time window for transfer of specimens to the thermocycler limit the advantage of large capacity and may present problems for laboratories not well staffed on all shifts. Therefore, many manufacturers have coupled automated nucleic acid extraction instruments with amplification and detection systems to create medium to high-throughput, fully automated NAATs. The PANTHER system (Gen-Probe) and the BD MAX (BD) are examples of fully automated and integrated systems designed to perform sample processing, nucleic acid amplification, and product detection all in one instrument (108,124,125). Although these systems have less requirements for human intervention and space (only one or two separate laboratory rooms are needed), proper cleansing is still needed to minimize the risk of contamination.

Advances in NAAT automation have enabled clinical laboratories to create value for decision making concerning e.g. infection control measures and choice of treatment. Many recent studies have demonstrated that the accuracy of automated and real-time NAATs to detect bacterial agents have been higher than that of traditionally used direct (antigen)immunoassay techniques (*e.g.*, group A streptococcus from throat swabs or *C. difficile* toxins from faeces) (126,127). Real-time assays have also been shown advantageous in rapid detection of organisms identified by specific cultures (*e.g.,* group A streptococcus

from throat swabs, group B streptococcus from vaginal/anal swabs, or toxin producing *C. difficile* from faeces) (4,116,126,128).

Enterotoxin producing *C. difficile* is a nosocomial and community acquired pathogen, causing clinical presentations ranging from asymptomatic colonization to self-limiting diarrhoea to toxic megacolon and fulminant colitis (115). According to Bartlett and Gerding, *C. difficile* infection (CDI) can be life threatening, with an attributable mortality of 6–15% (129). The clinical presumption of CDI should be proved by laboratory testing. The classic laboratory methods to detect *C. difficile*, *e.g.*, cell culture cytotoxicity neutralization assay and toxigenic culture have prolonged turnaround times, impairing their usability (117,130). When enzyme immunoassays (EIAs) emerged, they were widely adopted by many laboratories because of speed, convenience, and economical reasons. However, it has been demonstrated that immunoassays lack analytical sensitivity, which led in the era of NAATs for detection of *C. difficile* in clinical specimens (116,127,131-134). The sensitivity of real-time assays equals or exceeds the standard antigen or culture methods and the turnaround time for results is significantly shorter compared to culture-based method.

Perhaps the most significant impact of a molecular test on prevention of hospital-acquired infections (HAIs) has been the success of the methicillin (oxacillin)-resistant *S. aureus* (MRSA) screen. It has been demonstrated that infections caused by resistant strains, *e.g.* MRSA, have worse outcomes and higher associated costs than infections caused by sensitive strains, *e.g.* methicillin (oxacillin)-susceptible *S. aureus* (MSSA) (135-137). Many studies have shown that surveillance screening of MRSA and isolation of carriers can significantly reduce the incidence of nosocomial infections and be cost-saving (138).

Culture-based surveillance of MRSA may be inadequate for efficient infection control as the time required for final results can take several days. Studies show great promise of real-time NAATs for MRSA to simplify the process by providing same day results (139-141). Targeted and on-demand NAAT-based

screening of MRSA has altered presurgery prophylaxis and proved to be cost effective (142-146). According to Olchanski *et al.*, quick NAAT testing for high-risk patients was found to defeat the culture-based methods in terms of fewest infections and greatest potential cost savings (147). In another study by Roisin *et al.*, universal NAAT-based screening approach for MRSA on admission to hospital in an endemic setting shortened the time to implement isolation precautions (148).

There has also been considerable interest to apply real-time NAATs for testing of bacterial agents causing community-acquired pneumonia, especially *Streptococcus pneumoniae*, the most common agent associated with typical (lobar) community-acquired pneumonia. However, since colonization with *S. pneumoniae* under the age of five is fairly common, the NAAT assay for *S. pneumoniae* in this age group on a throat swab would be of limited value because this microbe is often a commensal in the upper respiratory tract (149,150). However, in adult patients' pneumonia is accompanied by bacteraemia in approximately 10–30 % of cases, which increases the mortality of pneumococcal disease (5). Thus, a quick and reliable diagnostic method to detect the causative agent of blood-stream infections (BSI) is essential.

Blood culture is the current "gold standard" of BSI diagnosis. The accurate identification of microorganisms and their portal of entry are central to the optimal management of BSI (151). Enrichment of microbes allows the possibility of evaluating comprehensively antimicrobial susceptibilities which has still not been paralleled by any other technique available to date (20). Blood cultures are currently performed with continuous microbial monitoring systems using fully automated instruments and incubators. The instruments detect microbial growth by the analysis of CO_2 release using fluorescent sensors (Bactec 9240; Becton Dickinson) or colorimetric sensors (BacT/Alert; bioMérieux, France) or, alternatively, by measuring pressure changes in the bottle headspace due to the consumption and production of gases (VersaTREK; TREK Diagnostic Systems) (20). After a positive signal, usually within 12 to 48

hours of incubation (152), a Gram stain is performed together with a preliminary evaluation of the antimicrobial susceptibility by disc diffusion or minimal inhibitory concentration (MIC) method directly from the blood culture bottle (153). Pathogens are still often identified by conventional biochemical tests which allow the identification of many pathogens commonly recovered from blood cultures within 16 to 24 hours; however, more time is often needed for final identification and antimicrobial susceptibility evaluation, especially when slow-growing pathogens such as yeasts or anaerobes are present (152,153).

During the last few years, various methods have been developed to optimize the detection of the etiological agent of BSI including fluorescence hybridization probes (154,155), pathogen specific and multiplex PCR (156,157), microarray (158), and MALDI-TOF MS (159,160). Despite the advantages of these assays many of them, however, comprise multiple assay steps to purify the microbes and/or to extract the target protein or DNA from the blood culture samples. Therefore, further simplification of these methods is desirable in order to reduce cost, labour-intensiveness, need for complex and expensive instrumentation, and expertise on molecular biology. Modern automatization of nucleic-acid based assays and development of detection technology have recently enabled interesting NAATs such as the FilmArray (bioMérieux, France) and the Verigene (Luminex, USA) systems for rapid multimicrobial detection from signal positive blood culture bottles (157,161).

In addition to NAATs that detect and identify pathogens from positive blood culture bottles, there are also NAATs that permit detection and identification directly from blood, serum, or plasma samples (20). According to Mancini *et al*., three types of detection strategies have been described, pathogen-specific assays targeting species- or genus-specific genes, broad-range assays targeting conserved sequences in the bacterial genome, such as the panbacterial 16S rRNA gene, and multiplex assays allowing the parallel detection of species- or genus-specific targets of different pathogens potentially involved in a certain infection type (20).

However, despite the remarkable technical advances of NAATs, *i.e.* short turn-around time, high sensitivity, and specificity, their widespread use in the diagnostics of bacteraemia is still limited by insufficient cost-effectiveness (20). More studies are needed to evaluate the clinical usefulness of NAATs as enhancing the performance to blood culture.

2.7 Small-scale point-of-care compatible platforms and applications

The idea of miniaturization of available technologies was created already by Richard P. Feynman in his famous lecture in 1959 (162). According to Dario *et al.*, the miniaturized systems in medicine and biology belong to one of the four areas of application, diagnostics, drug delivery, minimally invasive surgery, or neural prosthetics and tissue engineering (163). In clinical diagnostics, the recent developments in combining electronic and non-electronic functions by new mechanical, optical, fluidic, and electronic functionalities with already established NAAT applications has provided decisive advantages (75).

These miniaturized systems can be characterized by an integration of nucleic acid extraction, target amplification and detection onto a single small-scale platform. Such system incorporates many of the required steps of a typical room-sized laboratory on a small chip or cassette (19,23,164,165). The miniaturized and fully-automated systems offer the possibility to leave a clean lab environment, because of the incorporation of all necessary reagents on the device (75,166-170). Thus, contaminations are greatly reduced.

As defined by Ehrmeyer and Laessig, POC testing is something that occurs near or at the patient instantly or in a very short time frame (171). Until the 21st century, most existing POC tests consisted of immunoassays, namely agglutination, immunochromatographic and immunofiltration tests (172). During the first decade of the 21st century, however, non-immunological POC tests based on NAAT detection have become available (19,125,166,167,169,173). In molecular diagnostics, POC testing could significantly decrease the delay due

to specimen transportation which abrogates one of the key advantages of NAATs, namely, rapid turnaround time (TAT). Moreover, POC compatible NAATs enable tests that are accessible to personnel without specific laboratory medicine training allowing quicker delivery of results that directly influence the clinical decision (165).

There are two categories of on-demand sample-to-result NAAT available for molecular POC testing, small bench-top analysers, *e.g.,* Alere i (Alere), GeneXpert I, II and IV (Cepheid), Verigene (Nanosphere), Portrait (Great Basin), ePlex (GenMark), Illumigene (Meridian Bioscience, Inc) and FilmArray (BioFire / bioMérieux) (21,168,174,175), and hand-held single-use devises, *e.g.*, cobas Liat (Roche Diagnostics) (168), and GeneXpert Omni. In these, the sample preparation consists of suspending the specimen into sample buffer and transferring the suspended solution into a disposable, single-use cartridge or chip, which contains all the reagents required for the sample extraction and analysis and which are then processed in the automated instrument. Assay run time on POC compatible NAATs varies from 20 to 90 minutes depending on the instrument (21,168,174). Currently, as being compact systems, many of these instruments allow only one or few sample analyses at a time. Another limitation is the price of most on-demand NAATs.

As the number of different NAATs in microbial diagnostics is rapidly increasing, the need for proper evaluation studies to determine the assay's quality and usefulness in clinical laboratories are on the rise. According to Afshari *et al.*, some of the published evaluation studies have suffered from shortcomings, such as the application of an inappropriate gold standard or a non-convincing cost-effectiveness analysis (176). When evaluating a new test system, validation of the instrument and test performance, as well as clinical relevance, cost, and ease of use should always be considered (177). Therefore, a detailed protocol for the validation of new test methods should be established by the laboratory.

According to Espy *et al.*, after validation, external quality assurance programs are also necessary to demonstrate that a verified test continues to perform according to the laboratory's requirements (13). The follow-up procedures help ensure the consistency of the test results and that healthcare personnel remain competent to perform tests and report results (13).

3 AIMS OF THE STUDY

The aim of this study was to evaluate the rapid detection of clinically important bacteria by new small scale fully-automated NAATs. More specifically, the thesis contains:

- A performance and usability investigation of GenomEra MRSA/SA assay for routine use in a microbiological diagnostic laboratory enabling faster confirmation and reporting of negative and positive MRSA screening results (I).
- A performance and usability investigation of GenomEra *C. difficile*, BD MAX *C. difficile*, and GenRead C. difficile, for rapid and simple detection of toxigenic *C. difficile* (III, IV, VI).
- An evaluation of a one-step sample preparation of positive blood cultures for the direct detection of methicillin-sensitive and -resistant *S. aureus* and methicillin-resistant coagulase-negative staphylococci, as well as *S. pneumoniae* using the GenomEra MRSA/SA and *S. pneumoniae* assays (II, V).

4 MATERIALS AND METHODS

4.1 Bacterial isolates and clinical specimens

The list of bacterial isolates and clinical specimens used in the studies are presented in Tables 1–3. Two kinds of sets of samples were used: retrospectively collected culture collection isolates (n = 555), and consecutive clinical screening specimens (n = 2776).

Table 2. Samples analysed with the GenomEra CDX system

Bacterium and source	Number of isolates / specimens	Description of isolates / specimens	Reference	Time interval of collection
Culture collection	*555*	Retrospectively collected clinical and type culture collection isolates		
MRSA	304	Clinical isolates, known genotypes (*spa*, PFGE, and SCC*mec*)	I	1996–2010
C. difficile	15	Clinical isolates, known ribotypes and virulence genes	III	2012
S. pneumoniae	37	Type culture collection isolates	V	2013
Other isolates	217	Clinical isolates and type culture collection isolates	I, III, V	2012, 2013
Clinical specimens	*1616*	Prospectively collected clinical specimens (including pharyngeal, nose, groin and wound swabs, faeces, and blood cultures)	I, II, IV, V	2010–2014

Table 3. Samples analysed with the BD MAX system

Bacterium and source	Number of isolates / specimens	Description of isolates / specimens	Reference	Time interval of collection
Culture collection	*0*			
Clinical specimens	*302*	Prospectively collected clinical faecal specimens	IV	2014

Table 4. Samples analysed with the GenRead system

Bacterium and source	Number of isolates / specimens	Description of isolates / specimens	Reference	Time interval of collection
Culture collection	*0*			
Clinical specimens	*1160*	Prospectively collected clinical faecal specimens	VI	2014

Clinical screening specimens consisted of swabs (from pharyngeal, nose, groin, and/or wound) (Study I), blood cultures (Study II, V), and faeces (Study III, IV, VI), and were collected during 2010–2014 at hospital district of Vaasa (Finland) (Study, I, II, III, IV), Pirkanmaa (Finland) (Study I, II, V, VI), Varsinais-Suomi (Finland) (Study I, II), Pohjois-Pohjanmaa (Finland) (Study VI), and Grenoble (France) (Study VI). Sample analyses were performed at the central hospital laboratory of Vaasa (Vaasa, Finland) (Study I, II, III, IV), Fimlab Laboratories (Tampere, Finland) (Study I, II, V, VI), Turku University Hospital Laboratory (Turku, Finland) (Study I, II), Nordlab Oulu (Oulu, Finland) (Study VI), and Grenoble University Hospital Centre (Grenoble, France) (Study VI).

Culture collection isolates originated from clinical specimens collected during 1996–2013 (Study I, II, III, V) and a set of type culture collection isolates (Study V). All isolates were stored at -70 ^{o}C prior thawing and re-culture of the samples. The collections were situated at HUSLAB Helsinki University Hospital Laboratory (Helsinki, Finland) (Study I), National Institute for Health and Welfare (Helsinki, Finland) (Study I, III), and Vaasa central hospital laboratory (Vaasa, Finland) (Study I, III). Genotyping and ribotyping of isolates were performed at the National Institute for Health and Welfare (THL) as previously described (178-184).

4.2 Reference and conventional laboratory methods

All culture collection isolates and clinical specimens were cultured on a suitable media and incubated in appropriate conditions (Table 5). Colonies expressing typical morphology were identified using Gram-staining and biochemical tests as described in Table 5. 16S rDNA sequencing was used if species identification result was not clear (185) (Study I, II, and V). The antimicrobial susceptibility, particularly of the MRSA isolates, was tested according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (before 2012) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (from 2012) using the disc diffusion method (Oxoid and Mast, UK)

and Etest agar gradient diffusion method (bioMérieux, France) on Mueller–Hinton agar at 35°C (Study I, II) (186,187).

Table 5. Culture based reference methods

Target microbe	Culture medium	Source	Incubation conditions	Identification tests	Source
S. aureus (MRSA or MSSA)	Blood agar	BD	35±2 °C with 5% CO_2	API Staph	bioMérieux
	Chocolate agar	BD	35±2 °C with 5% CO_2	Staph ID 32	bioMérieux
	CHROMagar MRSA / Chrom ID MRSA	CHROMagar / bioMérieux	35±2 °C with ambient air	Tube coagulase	Labema
				SaSelect agar	Bio-Rad
Toxigenic *C. difficile*	CCFA	Oxoid	35±2 °C with anaerobic atmosphare	C. DIFF QUIK CHEK®	Techlab
	Fastidious anaerobe agar (FAA)	Lab M	35±2 °C with anaerobic atmosphare	Vitek MS	bioMérieux
S. pneumoniae	Blood agar	BD	35±2 °C with 5% CO_2	Optochin test	Oxoid
	Chocolate agar	BD	35±2 °C with 5% CO_2	Vitek MS	bioMérieux
	Fastidious anaerobe agar (FAA)	Lab M	35±2 °C with anaerobic atmosphare		

MRSA isolates were confirmed using either DNA-hybridization strip technology (GenoType® MRSA, Hain LifeScience, Germany), in-house PCR assay, based on amplification of *mecA* gene and *S. aureus* specific nuclease gene (*nuc*) (188), or by the Xpert MRSA Nasal test, detecting *S. aureus* protein A (*spa*) and the *mecA* sequences (Study I, and II). More precise isolate genotyping was performed at the National Institute for Health and Welfare.

Blood culture samples (Study II, V) were drawn from febrile hospitalized patients either in BacT/Alert FAN Aerobic (FA), Standard Anaerobic (SN), and Pediatric FAN (PF) bottles (bioMérieux, France) or in BACTEC™ Plus Aerobic/F, Plus Anaerobic/F, and Peds Plus/F (n027) bottles (Becton Dickinson, MD, USA). Blood cultures were incubated in the BacT/Alert 3D or BACTEC 9240 automated continuous monitoring systems according to the manufacturers' instructions until they signalled positive or for a maximum of 7 days, after which they were interpreted as negative. Signal-positive cultures were Gram

stained and pure cultured on rich universal agars (Table 5). Species identification was performed using conventional culture-based and biochemical methods, *e.g., Sa*Select™ identification agar (Bio-Rad, USA), tube coagulase, optochin susceptibility, Staph ID 32, Strep ID 32, Vitek®2, or Vitek MS (bioMérieux, France).

Toxigenic *C. difficile* was screened either by culture (Table 5) (Study III, and IV), by the immunochromatographic antigen C. DIFF QUIK CHEK® test (Techlab, USA) targeting *C. difficile*-specific glutamate dehydrogenase (GDH) (Study III, VI), or by NAAT assays other than those investigated; namely illumigene *C. difficile* (Meridian Bioscience Inc, USA), and IMDx™ *C. difficile* for Abbott *m*2000 (Abbott Laboratories, USA) (Study VI).

4.3 Nucleic-acid amplification techniques and sample preparation

4.3.1 GenomEra CDX system

GenomEra CDX (Abacus Diagnostica, Turku, Finland) is a computer controlled thermal cycler with a fluorometer capable of time-resolved and prompt fluorescence measurements (189). The instrument dimensions are 54 × 32.5 × 39.3 cm (depth, width, and height, respectively) and it weighs approximately 33 kg.

The GenomEra CDX uses disposable and optically transparent polypropylene reaction vessels, *i.e.,* test chips, (41 × 11 × 1.5 mm) that are specifically designed for the instrument and readily contains all the PCR reagents required for the analysis, including the polymerase, in dry form. Each chip also contains an internal amplification control of a non-naturally existing DNA sequence, which is used to monitor for the presence of inhibitors and the integrity of the dried reagents. In the beginning of an assay run, the test chips are irreversibly sealed to minimize the risk of cross contamination. Subsequently, the bacterial cells in the sample are disrupted by heat to release their genetic material into the reaction. The amplification takes place when the chips are moving between

a range of heated blocks of different temperatures during the run. The metallic background of the chips ensures efficient transfer of heat from the thermocycler to the PCR reaction and thus decreases the time required for the target amplification. In addition to the denaturation, annealing/extension and measurement blocks, there are blocks that are set to more extreme temperatures. The extreme-temperature blocks are used to further increase the speed of temperature change and to create a sharp thermocycling profile. The other functions of the GenomEra CDX instrument include the detection and identification of the inserted chips, and the homogeneous measurement of fluorescence. One to four chips can be analysed at the same time in one assay run.

The detection of the amplification products, which in this study were *S. aureus* specific, highly conserved, gene (yet unpublished) and the marker of methicillin resistance gene (*mecA*) (Study I, II), *C. difficile* toxin B gene (*tcdB*) (Study III, IV), and *S. pneumoniae* specific gene (yet unpublished) (Study V), is based on a proprietary technique called enhanced competitive hybridization where two partially complementary oligonucleotide probes are used. The label probes are labelled with a time-resolved lanthanide fluorescent label, such as terbium (Tb) or europium (Eu), while the other probes are labelled with a quencher molecule (190). In enhanced competitive hybridization, both oligonucleotide probes contain additional terminal nucleotides that have no counterparts in the other probe, but which can bind to the respective strand of the target nucleic acid. Accordingly, both probes bind to the target nucleic acid, which accumulates during PCR, more tightly than with each other. When bound to the target nucleic acid, the fluorescence of the label probe significantly increases. Furthermore, as also the quencher probe preferentially binds to the target nucleic acid and hence competes less for the label probe in the presence of the target, the detectable fluorescence increases and leads to a highly sensitive detection of even low amounts of the target nucleic acid.

End-point lanthanide fluorescence signals are measured in a time-resolved manner after completion of thermal cycling. Prior to measurement, all nucleic acids are denatured by heating the reaction mixtures for 10 s at 108 °C and for

60 s at 100 °C, after which the reaction vessels are transferred to the measurement block kept at 50 °C. Signals are first recorded after 2.0 s (Eu) or 4.8 s (Tb) and again after 120 s at 50 °C. The measurement settings were as follows: excitation wavelength, 340 nm; emission wavelength, 545 and 615 nm for Tb and Eu, respectively. The rationale behind measuring the signals twice is to obtain signals when the probes were denatured and when they were hybridized, allowing accurate analysis of results without needing to compare the signals with negative controls or with a baseline signal determined using real-time monitoring of PCR. The measurement of fluorescence is performed as a final step after the 45 cycles of the PCR amplification have been completed. The whole sequence takes approximately 50 minutes. The lanthanide chelate labels used in the assays are heat-stable and resistant against sample-induced interferences (190). The long-lifetime emission of the labels allows effective elimination of the background fluorescence (*i.e.*, autofluorescence) that originates from the samples. Using the time-resolved fluorometric measurement technique, the test chips are excited with ultraviolet light, upon which the labels fluorescence (106). By applying a short delay between the excitation pulse and the counting of the photons emitted by the labels, the autofluorescence can be excluded.

A dedicated software is used for the interpretation of the results. Qualitative assay results are reported directly after the run by the software in written (positive, negative, or inconclusive) format with numerical signal values from -15 (negative) to +100 (strong positive) for the target of interest (*S. aureus*, *mecA*, *tcdB* or *S. pneumoniae*). Borderline or so called 'inconclusive' results are obtained when the signal values settle between -5 and +5, in other words below the limit of detection (LoD) of the assays.

The GenomEra CDX system was used in this study with the following assay kits; GenomEra MRSA/SA Diagnose (Study I, II), GenomEra *C. difficile* (Study III, IV), and GenomEra *S. pneumoniae* (Study V). The sample preparation was performed as follows: readily enriched specimens such as blood

cultures or bacterial growth on solid media were diluted (25 µl of a liquid sample such as blood culture or one bacterial colony) into 1 ml of the GenomEra sample buffer followed by adding 35 µl of the sample suspension in the test chip. For the direct analysis of faecal specimens, 1 µl of stool was collected with a sterile loop and diluted in 1 ml of GenomEra sample buffer. Then, four hundred microliters of the sample solution was aliquoted into a second tube containing glass beads and was vortex-mixed for 5 minutes. In the last step, 35 µl of the sample suspension was added in the test chip.

4.3.2 BD MAX system

The BD MAX system (BD, USA) (previously known as the HandyLab Jaguar system; BD-HandyLab, Ann Arbor, MI) is a fully automated computer controlled benchtop molecular diagnostic system, which contains two integrated subunits; an extraction unit and two 12-lane microfluidic qPCR unit (125,191). The size of the instrument is 75.4 × 94 × 72.4 cm (depth, width, and height, respectively), and it weighs approximately 125 kg.

The extraction unit consists of a liquid handling head mounted on a robotic gantry and associated assemblies which allow cell lysis, nucleic acid extraction, and mixing of nucleic acid with master mix reagents. With no user intervention, after the extraction, the system dispenses the sample into a microfluidic chamber where real-time PCR amplification and detection are performed. The instrument can process and analyse 1–24 specimens per run. Individual barcode for each BD MAX Sample Buffer tube is scanned by an external barcode reader and verified against the system Work List by an internal barcode reader, ensuring traceability throughout extraction and PCR. The qPCR unit has dedicated multi-colour LED optics: including 475/520, 530/565, 585/630, 630/665, 680/715 nm excitation/emission wavelengths analysis. It allows qualitative, quantitative, and melt curve analysis.

The sample processing proceeds as follows: specimens are first introduced into Sample Buffer tubes according to manufacturer's instructions. Scanned barcode-labelled Sample Buffer tubes are then manually placed into a sample

rack. Sample information is entered via the keyboard, or by external barcode reader. Test selection is made using the BD MAX system software. The appropriate number of Unitized Reagent Strips (URS) needed for the run are placed in the sample rack and securely seated. Foil-sealed lyophilized extraction and PCR reagent tubes are snapped into the appropriate positions on each URS. A 24-lane BD MAX Microfluidic Cartridge is placed into a drawer located behind each respective sample rack. Once the run is started, automatic verification of samples, strips, and reagents begins, followed by the extraction and purification process. Sample lysis and DNA extraction take place in each URS. Sample lysis is done by chemical and physical reaction and the extraction is based on magnetic particle purification. DNA present in the sample is bound to magnetic beads which have been coated with a proprietary DNA affinity matrix. Extraction solution in the URS, is then prepared for PCR analysis. After extraction, the purified nucleic acids are mixed with master mix, including probes and primers. The instrument transfers the PCR-ready sample into the sample injection port of the appropriate lane on the BD MAX Microfluidic Cartridge. After all programmed samples are injected, the drawer containing the BD MAX Microfluidic Cartridge is drawn into the reader, where automated PCR amplification and detection are performed. The BD MAX allows amplification curve view in real time and supports multiple PCR technologies such as TaqMan®, hydrolysis probes, Scorpions®/Molecular Beacon, MGB Alert®, and SYBR® Green.

The BD MAX system was investigated using a Cdiff assay kit (Study IV). For the assay, 10 µl of stool (or 50 µl, when a liquid-based microbiology (LBM) tube was used for specimen collection) was collected with a sterile loop and transferred to a BD MAX sample buffer tube containing 1.5 ml of buffer, which was then sealed with a septum cap and vortexed for 1 min before being placed in the BD Max System URS rack with BD MAX Cdiff Extraction Tube and one BD MAX Cdiff Master Mix tube. One BD MAX PCR cartridge is also placed on the BD MAX for every 12 specimens tested. The automated extraction procedure uses achromopeptidase, an enzyme exhibiting strong bacteriolytic activity against Gram-positive bacteria (192), and magnetic-bead technology. Every

test includes a sample-processing control (SPC). During the extraction, 475 µl of the sample is extracted and eluted into 25 µl. After 50 to 90 minutes of extraction (depending on the number of samples), the eluate was automatically added to a master mix and inserted into 4-µl chambers. Real-time PCR is completed in less than 30 min (45 cycles of 30.3 s) in a microfluidic chamber system. The results are reported as positive, negative, or invalid. On-board micro valves automatically seal reaction chambers. The *C. difficile tcdB* signal is detected in the FAM channel, 475/520 nm. The internal control is detected in the channel, 585/630 nm.

4.3.3 GenRead system

The GenRead Instrument (Orion Diagnostica) is a small bench top instrument for isothermal amplification and real-time detection of nucleic acids. It weights approximately 3.6 kg, and has a size of 23 × 22.5 × 15 cm (depth, width, and height, respectively). The GenRead Instrument is a stand-alone, mains-power or Li-Ion battery operated *in vitro* diagnostic instrument capable of producing qualitative test results from various patient samples. The battery unit is charged automatically when the power cable is plugged in. The battery must be recharged periodically by connecting the external power supply for at least 1.5 hours. The complete charging time is 5 hours. The Orion GenRead instrument has an in-built computer with touch screen. It is operated by touching the virtual buttons in touch screen with fingers or a stylus. The screen can be used both with bare fingers and with gloves on. There is always multisensory feedback when a button is touched: the button will indicate the touching both visually, by changing appearance, and with an audible sound. It also provides the user with messages and prompts for performing the assays, and gives test results and error messages. The 15.1 × 9 cm (width, depth) touch screen is capacitive and has 800 × 480 pixels.

The Orion GenRead amplification and measuring unit consist of a temperature controlled 12-hole thermal block enabling isothermal amplification of target in reagent tubes. Fluorescence is measured via the tube bottom by confocal

fluorescence sensors for 1-12 reactions simultaneously in real-time. The instrument can be equipped with one to three sensors. Each sensor is capable of measuring two different emitting wave lengths simultaneously. The self-contained instrument is used together with Orion GenRead reagent kits for rapid detection of various pathogens and is suitable for use in laboratories of various settings and sizes. The GenRead assays use a proprietary SIBA® (Strand Invasion Based Amplification) technique for isothermal nucleic acid amplification (173). The technology is based on amplification of nucleic acids at a constant temperature of 40 °C. The probes are quenched conformational fluorescent probes, labelled with fluorophores such as Cy5 and ROX. The results are interpreted by special algorithms built into the device's software. The algorithm is encoded in a data matrix found on a barcode card in each kit. The data is transferred to the Orion GenRead by scanning the card with the instrument's barcode reader.

In this study, GenRead C. difficile assay kit was used (Study VI). For the detection of *tcdB* gene, a small amount of stool sample with a flocked swab was taken and transferred into a GenRead filtration vial consisting of 1.5 ml of lysis buffer. Three to five drops of the lysis buffer were squeezed from the vial to an empty micro-tube and the tube was heated in a heating block at 95 °C for 5 minutes. The heat-treated sample was mixed with reaction buffer, and 40 µl of the mixture was pipetted into a reaction tube containing freeze-dried reagents for the *C. difficile* assay and the IC reaction.

5 RESULTS

5.1 Rapid verification of MRSA using the GenomEra MRSA/SA assay (Study I)

The performance of the GenomEra MRSA/SA assay to detect various MRSA isolates was evaluated using 450 culture collection isolates. The culture collection consisted of 304 genotypically different MRSA isolates, including 68 different *S. aureus* protein A (*spa*) types, 51 different pulsed-field gel electrophoresis (PFGE) types and subtypes, and ten different SCC*mec* genotypes, as well as 146 non-MRSA isolates. The GenomEra MRSA/SA reported all MRSA isolates as MRSA-positive and all MSSA isolates as SA-positive only. In addition, all methicillin-resistant coagulase-negative staphylococci (MRCoNS) yielded *mecA*-positive and SA-negative results. Thus, the sensitivity and specificity of the GenomEra MRSA/SA assay was 100 % for MRSA confirmation from pure cultures incubated overnight.

One coagulase-negative *Staphylococcus* species from the pure culture collection, identified as *S. warneri* / *S. pasteuri* with the Staph ID 32 and 16S rDNA sequencing, however, repeatedly gave a weak SA-positive signal (between +19 and +35) by the GenomEra MRSA/SA assay when retested from different colonies on the same pure culture and after re-culture. Furthermore, this particular isolate was shown to be *nuc*- and *mecA*-negative in the reference *nuc-mecA*-PCR. Consequently, the specificity of the SA-target alone on GenomEra MRSA/SA was 99.3 %. Three other *S. warneri* isolates from the culture collection yielded SA-negative results.

Usability of the GenomEra MRSA/SA assay for daily use in a microbiological diagnostic laboratory was investigated with 145 clinical MRSA screening specimens that were cultured on selective MRSA media and yielding MRSA suspected growth after an overnight incubation. Of the 145 suspected colonies picked from chromogenic MRSA plates and analysed with the GenomEra

MRSA/SA assay, 125 were detected as MRSA-positive and 20 as MRSA-negative. Results were convergent with the conventional identification and susceptibility testing methods and with the reference MRSA PCR methods. Only one pure colony from the screening plate was needed to obtain MRSA verification by the GenomEra MRSA/SA assay. As the hands-on time was approximately one minute per sample and assay run-time 50 minutes, confirmation of MRSA was attained within 55 minutes by the GenomEra MRSA/SA assay. By contrast with conventional methods, the confirmation of MRSA took additional 16 to 24 hours. In this study, the overall PCR inhibition rate of the GenomEra MRSA/SA assay was 0.17 %.

5.2 Rapid detection of toxigenic *C. difficile* (Study III, IV, VI)

The performance of three automated NAATs, the GenomEra *C. difficile*, the BD MAX Cdiff, and the GenRead C. difficile assay, was evaluated for detection of toxigenic *C. difficile* from faecal specimens. Particularly, the aim was to assess the utility of these assays for daily use in a microbiological diagnostic laboratory enabling faster detection and reporting of negative and positive *C. difficile* (toxigenic) screening results.

5.2.1 GenomEra *C. difficile* assay (Study III, IV)

The estimated analytical sensitivity of the GenomEra *C. difficile* assay was five CFUs per PCR reaction, as the minimal detectable number of viable *C. difficile* cells in the spiked stool samples varied from 1.40×10^2 to 1.50×10^2 cells/µl. However, this was presumptively assessed, because only a limited number of replicates were used in this study. From the culture collection isolates (n = 33), all *tcdB*-gene containing *C. difficile* isolates (n = 14) were detected as toxin-positive by the GenomEra *C. difficile* assay. The *C. difficile* isolate not carrying toxin genes, and other clostridial species (n = 12), as well as non-clostridial isolates (n = 8) all yielded negative *tcdB* results with the GenomEra assay, as expected. The sensitivity and specificity of the assay from pure cultures was 100 %.

Apart from culture collection samples, the performance of the GenomEra *C. difficile* assay and utility in diagnostic microbiology laboratory was assessed with 612 clinical stool specimens. One hundred and fifty-eight (25.8 %) were considered true positive for toxigenic *C. difficile*. Of these, the GenomEra *C. difficile* assay detected 155. Two specimens were reported positive only by the GenomEra, remaining negative by toxigenic culture and by other NAATs. The sensitivity of the GenomEra *C. difficile* assay for the screening of toxigenic *C. difficile* was 98.1 %, specificity 99.6 %, positive predictive value (PPV) 98.8 %, and negative predictive value (NPV) 99.3 %. The PCR inhibition rate of the GenomEra was 5.3 % with faeces in conventional containers and 0 % with faeces in a LBM tubes, the FecalSwabs.

The hands-on time for the GenomEra *C. difficile* assay was approximately 1 minute for one sample and 3 minutes for four samples (the capacity of the instrument). The assay run-time was 50 minutes. Thus, the total turnaround time for one to four samples was less than one hour.

5.2.2 BD MAX Cdiff assay (Study IV)

The performance and usability of the BD MAX Cdiff assay was investigated with 302 clinical stool specimens. Of these, 79 (26.2 %) were considered true-positive for toxigenic *C. difficile*. The BD MAX Cdiff detected 74 positive specimens correctly. Three additional positive results were reported by the BD MAX, which, however, remained negative according to all other methods and, thus, were determined as false-positives. The respective sensitivity, specificity, PPV, and NPV were 93.7 %, 98.7 %, 96.3 %, and 97.8 % for the BD MAX Cdiff assay. The PCR inhibition rate was 4.4 % with faeces in conventional containers and 0 % with faeces in FecalSwabs.

Hands-on time for analysing 1 to 4 specimens was 1.5 to 3 minutes, and for 24 specimens, 10 minutes. The assay run time for 1 to 24 specimens was 85 to 135 minutes.

5.2.3 GenRead C. difficile assay (Study VI)

In this study, a total of 1160 faecal specimens were analysed with the GenRead C. difficile test. One hundred and eighty-four specimens (15.9%) were considered as true positives for toxigenic *C. difficile*. A total of 11 false positives (0.9%) and 16 false negatives (1.4%) were reported with the GenRead C. difficile. The overall sensitivity of the GenRead assay was 92.0 %, specificity 98.9 %, PPV 94.4 %, and NPV 98.4 % as compared to the comparative methods. A total of 45 invalid results (3.9 %) were obtained with the GenRead test system during the study.

The precision of the GenRead C. difficile test system was evaluated by a reproducibility study, where the same sets of blind coded samples were tested at three separate laboratories. During a five-day testing protocol, five samples containing various amounts of toxigenic *C. difficile* or *C. sordellii* cells (in three replicates) were analysed twice per day by two laboratory technicians. A total of 449 samples (99.8 %) were in total agreement between the three laboratories. Only one sample was reported as false positive (in one laboratory) due to a probable contamination caused by a user.

The hands-on time with the GenRead C. difficile assay was approximately 5 minutes per one sample. The assay runtime varied from 15 to 55 minutes depending on the concentration of the *tcdB* gene in the specimen.

5.3 Rapid detection of *S. aureus*, the marker of methicillin-resistance, and *S. pneumoniae* in blood cultures using the GenomEra CDX system (Study II, V)

In these studies, a total of 859 blood culture samples were analysed, including 835 positive bottles containing Gram-positive cocci in clusters (n = 419) (Study II), Gram-positive or poorly stainable cocci either in chains or in diploid form (n = 110) (Study V), and other bacterial forms (n = 361) (Study II), as well as 24 signal- and growth negative bottles (Study II).

In total, nine (1.2 %) MRSA, 159 (20.4 %) MSSA, 133 (17.1 %) MRCoNS, and 97 (12.4 %) methicillin-susceptible CoNS (MSCoNS) isolates were identified by routine methods from the bottles containing Gram-positive cocci in clusters. The GenomEra MRSA/SA assay detected 158 MSSA and all nine MRSA strains, yielding clinical sensitivities of 99.4 % for detection of MSSA and 100.0 % for MRSA. Of the 133 MRCoNS positive samples, 132 were detected by the GenomEra assay, based on a positive *mecA* signal and a negative SA signal, yielding a sensitivity of 99.3 % for the presumable detection of MRCoNS. The one false negative MSSA and the one false negative MRCoNS results were obtained from two specimens containing polymicrobial growth. Analysis of the cell concentration in the polymicrobial samples revealed that the concentration of both MSSA and MRCoNS was suppressed to less than 1:1000 of that of the competing species and hence fallen under the LoD of the GenomEra assay. For the detection of *S. aureus* and the marker of methicillin resistance, the minimum detectable number of viable cells using the GenomEra MRSA/SA assay was 4×10^4 CFU / ml. Thus, the estimated analytical sensitivity for intact MRSA cells was 30 CFU per PCR reaction.

The specificity of the GenomEra MRSA/SA assay was 99.8 % for MRSA, 100 % for MSSA and 100 % for MRCoNS. One false positive MRSA result and one weakly reactive result reported as an inconclusive 'MRSA borderline' result were seen from two specimens, one containing *Serratia marcescens* (strong positive) and the other containing *Streptococcus pyogenes* (weakly reactive).

The sensitivity and specificity of the GenomEra *S. pneumoniae* assay with 90 culture collection isolates, including 37 known *S. pneumoniae* (18 different serotypes) and 53 non-*S. pneumoniae* isolates, was 100 %. Of the 110 blood culture specimens, *S. pneumoniae* was recovered and identified from 46 specimens (41.8 %) by the conventional culture based methods. Using the GenomEra *S. pneumoniae* assay, all 46 *S. pneumoniae* positive blood culture specimens were detected within 55 minutes after the preliminary Gram-staining. The remaining 64 blood culture specimens yielded a growth of various streptococci other than *S. pneumoniae*, enterococci, and other bacteria, and

were all negative by the GenomEra assay. These results indicated a 100 % sensitivity and a 100 % specificity for the GenomEra *S. pneumoniae* assay.

The hands-on time for one sample was less than 1 minute and for four samples approximately 3 minutes with both GenomEra assays. The assay runtime was 50 minutes and thus, the total turnaround time with the GenomEra assay, including extraction, for up to four samples (the capacity of the instrument) was approximately 53 minutes. The total PCR inhibition rate of the GenomEra assay from blood culture samples was 0.6 % (6 / 859).

6 DISCUSSION

Clinically important bacteria such as *S. aureus*, toxigenic *C. difficile*, and *S. pneumoniae* are responsible for a significant number of infections in many healthcare facilities and in community, resulting in excess morbidity and healthcare costs (5,6,115,136,137,193-198). To address this challenge, healthcare organizations have implemented various infection control strategies (199,200). According to Zimlichman *et al.*, prevention through education is the most used strategy worldwide for HAI and healthcare-associated infection (HCAI) control (10). Other implemented measures are reinforcing hand washing, environmental cleaning practices, Antibiotic Stewardship Programs (ASPs), and improved communication and epidemiological surveillance systems (10). However, to efficiently control HAIs and HCAIs, clinicians and health care workers need to act quickly, which implies gathering all relevant information about the infection as soon as possible. Clinical microbiology laboratories play a key role here, enabling the early identification of infectious aetiology agent, characterization of possible antibiotic resistance patterns and recognition of outbreaks.

6.1 The benefits and disadvantages of automated NAATs in MRSA screening

As stated in chapters 1 and 2.6, conventional laboratory techniques such as culture typically require several days for the identification of clinically important bacteria until fully completed. Moreover, if conventional phenotypic susceptibility tests are used alone for the detection of resistant bacteria such as MRSA without NAAT confirmation, additional problems may be encountered. For example, borderline oxacillin (methicillin)-resistant *S. aureus* (BORSA or MOD-SA) isolates, lacking the clinically important *mecA* or *mecC* genes, may be misinterpreted as MRSA, due to their elevated MIC values to oxacillin and/or cefoxitin (201,202). In addition, some clinical MRSA isolates have shown to be *in vitro* oxacillin-susceptible while containing the *mecA* gene (203,204). Correspondingly, these OS-MRSA isolates may be misinterpreted

as MSSA, due to their reduced MICs to oxacillin or cefoxitin, if phenotypic tests are used without NAAT confirmation.

Concurrently, advances in NAAT have made it possible for diagnostic information to have a greater impact on decisions for isolation, treatment, and care (113). However, effective use of molecular diagnostic technology requires good understanding regarding the scope, suitability, and performance of the NAATs, as well as critical interpretation of the test results.

In the first study of this thesis, the performance and utility of an automated small scale NAAT, the GenomEra MRSA/SA assay, utilizing a dual-target detection of *S. aureus* specific and the *mec* genes (at that time only *mecA*), for MRSA screening in clinical microbiology laboratory was investigated. Implementation of a fully automated "plug-and-play" NAAT platform in a laboratory lacking special facilities for molecular diagnostics was surprisingly simple. Moreover, due to the small size of the GenomEra CDX instrument it could be easily set up anywhere in the laboratory and relocated into a new place if needed. The assay itself is straightforward to perform as the hands-on time with the GenomEra MRSA/SA assay is less than 1 minute. The capacity of one GenomEra instrument proved to be 32 sample analyses (4 samples per one assay run) within one 8-hour workday. Thus, being adequate for the laboratories performing up to approximately 8,000 (on Monday to Friday) to 11,000 (on every day) analyses per year.

The GenomEra MRSA/SA assay demonstrated a high performance (100 % accuracy) by detecting the vast variety of genotypically differing MRSA strains from culture collection isolates and not yielding any false-positive MRSA results. However, since the dual-target approach is known to provide false MRSA-positive results when detecting simultaneously the *mecA* gene from MRCoNS and the *S. aureus* specific gene from MSSA in specimens containing mixed growth of MRCoNS and MSSA (205), the suitability of the GenomEra MRSA/SA assay for clinical purposes was investigated using a dual-step algorithm; culturing the specimen on a MRSA selective media followed by NAAT

confirmation using the GenomEra assay. This procedure permitted the detection of MRSA from patient samples within 24 hours. Consequently, MRSA-positive results could be reported to clinicians one day earlier with the GenomEra MRSA/SA assay than using conventional culture-based identification and phenotypic susceptibility testing methods. However, a major limitation of this two-step approach is that the overall sensitivity depends on the sensitivity of the different screening agars (206-210).

To overcome the limitation related to the performance of the preceding culture and to further reduce the time to MRSA detection, there is an alternative approach using NAAT; targeting the acquired right extremity sequence of the diverse genomic island complexes known as the staphylococcal cassette chromosome (SCC*mec*) including the chromosomal sequence of the open reading frame X (*orfX*), in combination with *S. aureus* specific marker and the mediator of methicillin resistance genes (*mecA* and *mecC*) (139,140,169,210-214). Assays using this approach are specifically designed for direct screening of MRSA from swabbed samples without any preliminary enrichment steps (139,140,169,210,212,213,215,216). Benefits of such direct NAAT testing is the superiority in speed, total turnaround time varying from one to five hours (depending on the system), and usage simplicity with highly automated systems as compared to conventional methods. Disadvantage, on the other hand, is that direct molecular screening is more costly than conventional culture and, for this reason, many clinical laboratories opt to use the culture-based screening. However, there are already few studies evidencing the clinical cost-effectiveness of direct NAAT-based MRSA screening (217,218).

Importantly, though, critical limitations related to both false-negative and false-positive results were reported shortly after the launching of NAATs targeting the SCC*mec-orfX* junction (209,212,215,219-223). False-negative results are related to the highly variable SCC*mec-orfX* junction in *S. aureus*. Sissonen *et al.* stated in their study that when using the assay, one has to bear in mind the possibility of mutating MRSA strains and to use regularly some other, strictly MRSA-specific test, particularly in low MRSA endemic regions,

like in Finland (215). The second issue regarding false-positive detection of MRSA can occur due to the *S. aureus* isolates which do not have a functional *mecA* gene but are detected by the primers targeting the SCC*mec-orfX* junction (220). For these limitations, dual-target detection of *S. aureus* specific gene and the *mec* genes is highly recommended in regions where the prevalence of MRSA is low but genetic variation in circulating isolates is high, *e.g.* in Nordic countries (141,212,223-225). This is because the *S. aureus* specific and the *mecA* genes do not variate as much as the SCC*mec-orfX* junction and currently used primers and probes are able to detect most, if not all circulating or emerging MRSA variants (178,180,181,226-230).

The dual-target detection utilized by the GenomEra MRSA/SA could also be used for direct screening of MRSA in low endemic settings. No performance data has yet been published for the direct screening of MRSA using the GenomEra MRSA/SA assay, but preliminary data shows that the assay provides a high NPV but lacking some specificity. For the direct detection of MRSA, the hands-on time with the GenomEra MRSA/SA is approximately 5 minutes and the total turnaround time an hour. However, if all screening samples were analysed by direct detection the capacity of the GenomEra instrument would be inadequate. The two-step MRSA screening algorithm, which was investigated here, has been shown to be more cost-effective approach in low endemic MRSA settings than a NAAT-only approach (217,218,231).

Despite the excellent performance of the GenomEra system, though, emerging new MRSA variants, such as the $MRSA_{LGA251}$ isolate found in Europe, containing the *mecC* gene and the new type of SCC*mec* cassette (type XI) (57), pose diagnostic problems with the potential risk to be misdiagnosed as MSSA by NAAT targeting only the *mecA* gene (232). This may lead to adverse consequences for patients. Thus, after implementation of any NAATs it is necessary to continue to monitor the performance of the assay, equipment, and reagents. Recently, the GenomEra MRSA/SA assay were updated to include both the *mecA* and the *mecC* gene targets to detect the new *mecC* MRSA variants, too. Since this diagnostic update was not available during the studies, all BORSA

isolates analysed here were confirmed later, just in case, using the *mecC* PCR assay (data not shown).

An interesting feature of the GenomEra assay is that it can be performed without any sample purification steps for many different specimen types including blood cultures. This is mainly due the proprietary, heat-stable and sample-induced interferences resistant, label technology and robust DNA polymerase (106). Therefore, the GenomEra platform is suitable for many routine applications. Due to the lack of labour-intensive and hence costly sample preparation steps the GenomEra MRSA/SA assay was further investigated for rapid detection of staphylococci in blood cultures.

6.2 The impact of NAATs in detecting bacteria from blood and blood cultures

As pointed out in chapter 2.6, the rapid and reliable detection of microbes in severe BSI is essential (2,152,233-235). Kumar *et al.* have reported that for each hour of delay in the administration of effective antimicrobial therapy there is a 7.6 % increase in mortality (236). In regard to staphylococci, bacteraemia caused by *S. aureus* is known to associate with high morbidity and mortality rates whereas CoNS in blood cultures originated often from foreign body colonization such as catheter or cannula or refer to contamination (237-243). Thus, a reliable differentiation of *S. aureus* from CoNS, in signal-positive blood culture bottles is essential. In addition, the rapid detection of antibiotic (methicillin) resistance is significant in order to reduce the risk of inadequate empirical antimicrobial therapy (244-246).

As compared to conventional methods, significant advantages were achieved with respect to speed and accuracy of staphylococci detection from blood culture samples by the GenomEra MRSA/SA assay. The culture-based methods and susceptibility testing took 16–48 hours for final identification of MSSA, MRSA or MRCoNS, whereas the results were obtained in only 55 minutes with the GenomEra. In addition, since the *mecA* gene itself is very rarely found in

species other than staphylococci (247), the GenomEra MRSA/SA assay provided assistance in the rapid detection of MRCoNS in blood cultures using positive *mecA* and negative SA amplification signals as an indicator. Similar observation has been made in another study with the Xpert MRSA/SA blood culture assay targeting *mecA*, *spa* and SCC*mec* genes (248). Although determining the significance of CoNS in blood culture can be difficult, its rapid detection may hasten the detection of nosocomial bloodstream infections of paediatric (249), immunocompromised and haematological (250) patients with a central venous catheter. Among those patient groups the most common pathogen is usually CoNS, of which over 80 % may be methicillin-resistant (249). Accordingly, the GenomEra MRSA/SA assay is able to improve the diagnostics of bloodstream infections caused by staphylococci by decreasing the total turnaround time and thus permitting an earlier implementation of appropriate antimicrobial treatment.

Despite the high performance of the GenomEra MRSA/SA assay, two false-negative (n = 2/261) and two false-positive (n = 2/520) results were encountered in the current study. Of the false-negatives, one was for MSSA and one for MRCoNS. These were due to polymicrobial growth resulting in suppressed growth of the species of interest, presumably due to a lower initial concentration, poorer condition of the cells, or unfavourable growth in the given conditions as compared to the competing species. Although the average concentration of bacterial cells in signal-positive blood monocultures is remarkably high (251) and exceeds the detection limit of NAAT, mixed cultures encompass an inherent risk that the positive signal originates from the growth of a species other than the one of interest. In case of suspected polymicrobial growth, *e.g.*, based on the Gram-stain, thorough mixing of the blood culture bottle prior to sampling is recommended to enhance the target microbe detection by ensuring a maximal cell concentration in the sample. The *ex vivo* behaviour of microbes in multimicrobial infections is, however, difficult to anticipate, but it probably would affect similarly all NAAT systems (252).

Of the false-positives, one sample yielded MRSA-positive result and one sample weakly reactive MRSA-inconclusively positive result. However, both of these results were from samples, which would not normally be analysed by this assay, as neither of the sample bottles contained Gram-positive cocci in clusters but only Gram-negative rods (*S. marcescens*) and Gram-positive cocci in chains (*S. pyogenes*). Both species are known to produce extracellular DNA nucleases (253,254), which may cause interference with the result interpretation by partially cutting the oligonucleotide probes in the test. Neither of the isolates yielded MRSA positive result after re-runs from isolated colonies, which supports the hypothesis of false positive result by nuclease activity. False-positive MRSA results from blood cultures can lead to an inappropriate patient care, *e.g.*, unnecessary precaution measures and the use of suboptimal ineffective antimicrobial therapy. Therefore, pathogenic specific NAAT assay should be run only after Gram-staining. In addition, MRSA positive results by NAAT should be confirmed by other laboratory methods when mixed blood culture samples are suspected.

The utility of pathogen-specific assays in the diagnostics of BSI is limited, though, due to the high variety of potential pathogens (255). Additional microbial targets are required to get full benefit of NAATs in sepsis diagnostics. Using a method that permits the detection of several significant pathogens would save investment costs and training expenses of the laboratory personnel. For the GenomEra CDX, another assay for the diagnostics of BSI is available, namely the GenomEra *S. pneumoniae* assay. Since *S. pneumoniae* is one of the most significant pathogen causing non-invasive and invasive infections (7) its rapid and reliable detection, especially from blood culture of seriously ill patients, is essential. The GenomEra *S. pneumoniae* demonstrated a highly accurate and reliable differentiation of all tested *S. pneumoniae* isolates from other streptococci. The GenomEra *S. pneumoniae* assay provided a clear benefit over routinely used non-NAAT identification methods by reducing the time to species-specific identification in blood cultures significantly (<1 hour vs. 18–48 hours), yet with equivalent diagnostic sensitivity and specificity. A straightforward (hands-on time 1 minute for one sample) sample processing

for *S. pneumoniae* assay, identical to MRSA/SA assay, and the possibility to analyse different assays simultaneously in separate sample slots, makes the GenomEra CDX platform an attractive alternative in specific BSI diagnostics.

The cost-effectiveness of various diagnostic methods or their combination has become an important topic. The use of a pathogen-specific NAATs following Gram-staining of positive culture bottle samples has been suggested to be more practical and cost-effective in blood culture diagnostics than the use of multiplex PCR or broad-range amplification followed by sequencing analysis (20). It should be noted though, that the performance of combination diagnostics depends on the sensitivity and specificity of the screening method, in this case, the blood culture system. It is well known that some bacteria, *e.g. S. pneumoniae*, may grow very poorly or not at all in blood culture bottles, especially when patients have received antibiotic treatment prior to specimen collection (256-258).

Implementing NAAT-based assays for the detection and identification of bacterial pathogens directly from blood, plasma, or serum samples would allow a significantly shorter turnaround time (within 6–8 hours) compared to classical blood culture-based methods (48–72 hours) (20). The combination of universal PCR targeting conserved regions with sequencing (259,260), or hybridization (261) has been applied for the direct detection of bacterial pathogens from blood samples. This approach may allow the direct detection of any cultivable or non-cultivable bacterial pathogen, but it carries risks, too, such as contamination of samples with microbial DNA present in extraction or PCR reagents leading to false-positive results (262).

Promising approaches for routine use in clinical laboratories are multiplex real-time NAATs in combination with DNA microarrays. These tests allow more specific and rapid identification of pathogens directly from a biological sample (263-265). However, most of these NAATs comprise multiple manual or automated sample preparation steps, rendering the tests laborious, expensive, and/or requiring special skills in molecular biology. In addition, some of

these tests have a relatively poor sensitivity as compared to the blood culture method (263).

According to Tomas *et al.*, the detection of circulating microbial DNA (DNAemia) with rapid NAATs does not necessarily indicate the presence of a viable microorganism responsible for a given infection (266). The high sensitivity needed for the diagnosis of sepsis may increase the risk of false-positive results due to carryover contamination or due to the detection of environmental DNA contaminating the blood sample. Moreover, DNAemia may be the footprint for transient bacteraemia not related to infection (266), or it may be related to the persistence of circulating DNA still detectable several days after successful anti-infectious therapy has been completed (267). Consequently, conventional blood culture systems have remained the standard methods for the enrichment and identification of BSI pathogens. Thus, improving the species detection from signal-positive blood culture bottles may increase the probability of favourable clinical outcome, provided that the overall blood culture process is optimized and the results are hastily reported to physicians. In addition, quick delivery of the culture bottles in the incubator cabinet, and rapid reaction to the growth signal are significant factors in the process of BSI diagnostics.

6.3 Utility of automated NAATs for the screening of toxigenic *C. difficile* in faeces

Besides MRSA screening and NAAT assay application in BSI diagnostic, rapid detection of pathogenic microbes and/or toxin and other virulence markers in faecal matrix has been of general interest. However, since stool contains a lot of different substances inhibitory to NAATs, as well as an abundant microbial normal flora disturbing the detection of pathogenic microbes, the performance and accuracy of an assay is highly emphasised. In this study, the direct detection of toxin-producing *C. difficile* in faecal specimens was assessed using three different NAAT assays, the GenomEra *C. difficile*, the BD MAX C diff, and the GenRead C. difficile. None of the fore-mentioned tests actually detects the *C. difficile* but rather the conserved region of the *tcdB* gene specific for the toxigenic form of the species. Although some of the early assays detected *C.*

difficile toxin A (TcdA or the *tcdA* gene) exclusively, it is now generally recommended that assays detect toxin B (TcdB or the *tcdB* gene) or both TcdA and TcdB, since TcdA-negative, TcdB-positive disease-causing strains and outbreak of severe infection caused by such strains have been well documented (115). Moreover, TcdB is referred to as the cytotoxin because it is 100- to 1,000-fold more potent *in vitro* in cultured cells than TcdA (268).

In overall, all the NAATs demonstrated excellent sensitivity and specificity in our sample material consisting of antibiotic associated diarrhoea patients, even though there were a few discrepant results in which the infectious agent was detectable by the NAATs only and not by culture and vice versa. These findings are consistent with previous studies investigating the performances of various NAAT assays for the detection of toxigenic *C. difficile* in faeces (127,128,269-272). While significant differences in sensitivity between the NAATs and culture could not be obtained, the total turnaround time for detection of toxigenic *C. difficile* decreased significantly with NAATs (0,5–2,5 hours) as compared to toxigenic culture (48–72 hours).

In comparison with the commonly used POC-compatible toxin A/B immunoassay (IA) test, on the other hand, NAATs improved significantly the detection of toxigenic *C. difficile* (P value <0.0001), a finding that is known from earlier reports (116,127,134,269-273). When toxin A/B IA tests are used as stand-alone tests in clinical microbiological laboratories, many clinical presentations compatible with CDI may remain without confirmation or may be erroneously considered *C. difficile*-negative. Crobach *et al.* have suggested though, that the detection of CDI with IA or EIA tests could be improved using 2- to 3-step diagnostic algorithms (274). These approaches combine a preliminary IA screening test with more sensitive test such as toxin-detecting EIA, NAAT, or culture. However, recent studies have demonstrated that the sensitivity of 2- to 3-step algorithms may still be as low as 41–68 % (275). Moreover, some IA tests are prone to subjective result interpretation, unlike the automated NAATs which use accurate detection optics in combination with mathematic signal to noise detection algorithms.

The level of sensitivity needed for CDI diagnostic testing is not yet clear, as stated recently by Stellrecht *et al.* (191). It has been assumed that sensitive methods, such as culture or NAATs, are not able to discriminate between CDI and asymptomatic colonisation. Asymptomatic carriage of toxigenic *C. difficile* can be common among children and elderly inpatients, and among patients in extended care facilities *i.e.* nursing homes (276-279). However, the detection of asymptomatic carriage may have relevance, if the diagnostic purpose is to investigate the transmission of *C. difficile* in different health care settings (276,279). Thus, good practice requires careful consideration of testing indication, and in the case of CDI, attention should be paid to performing NAATs on symptomatic patients only (275).

Due to the high cost of NAATs compared to conventional culture or IA and EIA tests, the issue of cost-effectiveness has been raised by some experts and administrators (280,281). However, studies are beginning to emerge to address the impact on pharmacy costs and length of stay in isolation, with favourable results. For example, Peterson and Robicsek, and Brecher *et al.* highlighted that although the cost of a test is low, it is of little value if the result is inaccurate and has to be repeated many times over several days to get an accurate result (275,282). In addition, Tenover *et al.* created recently a hypothetical model based upon testing of 1,000 patients, assuming a 10 % prevalence of CDI and using published performance characteristics of various test methods (283). Their observation was that NAAT testing alone, compared to a toxin EIA alone and to various two-step algorithms using IA detection, resulted in the largest number of patients with disease who would be placed in isolation within 24 hours. Furthermore, NAAT testing resulted in the largest number of patients who would be removed from isolation more quickly because the rapid NAAT would detect them more rapidly, and in the smallest number of patients with false-negative tests who would not be placed in isolation and consequently who would continue to spread the organism within the hospital environment (283).

6.4 The pros and cons of automated NAATs in clinical microbiology diagnostics

Short hands-on and total turnaround times with high performance are clear advantages of automated NAATs as compared to conventional culture-based method and IAs or EIAs. However, there are numerous different NAAT platforms on the market with different characteristics. Of the three platforms investigated here, the BD Max is designed for laboratory use only requiring space and expertise in molecular biology as well as knowhow for robotics. Thus, it was the most complicated system to use. However, the capacity of the instrument was 24 samples per one 2.5 hours run, making it suitable for medium to large-sized laboratories performing 24 or more sample analyses per day. In addition, the BD Max platform proved to be the most flexible system enabling "in-house" or user-design assays to be used on the system along with the commercially available assays provided by BD. Moreover, the current assay menu was the widest (numerous multiplex assays for bacterial and virus targets). The GenomEra, on the other hand, proved to be less laborious than the BD Max system. Due to the simple sample preparation without manual DNA extraction steps, closed test chip, user-friendly application, fast turnaround time (50 minutes), and scanty space requirement make the GenomEra platform suitable for smaller laboratories lacking room for larger PCR systems as well as for POC settings. However, the GenomEra system allows only 4 samples to be analysed per one assay run, which limits its usefulness, *e.g.*, during epidemics.

The GenRead system, based on isothermal technology, proved to be an interesting platform being suitable not only in microbiology laboratories but in POC facilities and resource-limited settings, as well. Being self-contained and only 0.0078 cubic meter in size and weighting ~3.6 kg, the GenRead instrument can easily be carried, *e.g.*, from a laboratory to wards or clinics. Moreover, since the instrument can be operated up to 8 hours using battery-power, it can analyse samples even during transport. The battery-powered technology minimizes also the problem regarding inconsistent or non-existent local elec-

trical sources, particularly in resource-limited settings. The sample preparation and result interpretation of the GenRead system is simple, enabling diagnostic aid even in settings where little decision support is available. The test results are shown on the instrument touch screen within 15 minutes as its best, in a clear and unambiguous form as positive, negative, or in case of inhibition, as invalid. Transportable devices that are easy to use and interpret allow diagnostic laboratories to create mobile NAAT units where sample analysis can be initiated directly after specimen collection.

As discussed by Peeling and Mabey, rapid and reliable POC tests for infectious agents could save many lives, especially in developing countries, by increasing access to proper diagnosis and treatment (284). Similarly, Buchan and Ledeboer stated in their recent review, that rapid and fully automated on-demand or single-test formats have potential to affect health-care decisions at its earliest stage (12). They also state that the total cost of a patient care can be reduced by rapid diagnosis, even though the automated or single-test format are often more expensive than batched testing. iNAATs enable the construction of cheaper instruments that do not require stringent control or rapid changes of the temperatures like PCR. Such technologies allow more cost-efficient NAAT POC-compatible platforms that could be more affordable also in developing economies.

In addition to POC single-test analyses, the GenRead system can be adapted for use in high-throughput screening at centralized laboratories. The instrument enables four batches of 12 sample runs (in total 48 samples) in less than 3.5 hours and five batches (in total 72 samples) in less than 4.5 hours, with minimal space and energy requirement. The throughput with the GenRead system resembled the fully automated yet more complicated high-throughput NAAT systems. However, the hands-on time increases significantly if more than 24 samples are processed per day due to the pre-handling steps required for the GenRead C. difficile assay.

Despite the benefits of NAATs and iNAATs in clinical diagnostics, these techniques still pose some challenges, as stated by Bunham and Carrol (115). Some concerns relate to the biology of a bacterium and how the detection of, *e.g.*, genes that encode toxins correlate with expression of toxins. Also, given the heterogeneity of many microbial species, will strains emerge that are not detected by a particular assay? The first point is more challenging, but the answer to the second question is a systematic external and internal quality assurance. The performance of each device and test must be monitored routinely to ensure the continuous performance of NAATs. Moreover, as some sample materials, *e.g.* stool, might be a challenging matrix for NAATs (12) and since PCR inhibition is known to have a notable effect on the diagnostic performance of the NAATs (29), all assays should have built-in controls (ICs) to assess inhibition. All assays investigated here have sample-relating ICs. The level of inhibition from fresh faeces with the GenRead C. difficile assay was approximately 4 %, with the GenomEra *C. difficile* assay 4.4 % and with the BD Max C. diff 5.3 %. By eliminating the problems due to inhibitors, NAATs are clearly superior to the conventional test methods. For example, by diluting the sample prior NAAT assay-run, inhibition may be avoided. Here, the level of PCR inhibition was decreased into 0 % when faecal specimens were collected (diluted) into LBM tube.

7 CONCLUSION AND FUTURE CONSIDERATION

Molecular techniques are developing and becoming more and more useful and usable in laboratory and POC settings. Along NAAT automation and robotics, more specimens can be analysed in clinical laboratories with less hands-on time. Accordingly, total turnaround times of many microbiological analyses become shorter and the test results are more readily available to clinicians earlier. Moreover, movement towards NAAT-based POC testing, being pursued through simplification and miniaturisation of the testing process and test platforms, allow more rapid sample-to-results answers thereby enhancing disease management and patient treatment even further (12,284,285). POC compatible and low-cost iNAATs provide access to state of the art diagnostic support even in settings where health-care infrastructure is minimal.

At present, the number of different commercialized NAATs is plenitude, each having its own benefits and shortcomings, as well as optimal indications for usage. Although NAAT-based POC testing has shown to reduce unnecessary test orders and the length of hospital stay, as well as shorten the time interval to appropriate therapy (286), the lack of published studies objectively examining quantifiable outcomes related to the use of POC testing is still evident (286). Thus, additional studies are needed to evaluate the utility and usability of POC compatible, particularly, mobile NAATs in clinical microbiology diagnostic. In this study, three NAATs, one suitable for mobile diagnostics, were investigated for the detection of three different pathogenic bacteria. Since, the development of new technologies is very intense, it is likely that additional tests will become available for the platforms tested here in the near future, as well as more new devices.

A so-far underutilized potential of one of the systems tested in this study, the GenomEra assay, concerns direct testing of body fluid or abscess specimens. In preliminary studies, the assay has proven to be a promising aid in the rapid

detection of *S. aureus* and *S. pneumoniae* from liquor, and joint fluids. Consequently, this should be studied further. Another interesting study project would the utilization of the portable GenRead device in the rapid detection and control of various outbreaks in health-care settings. However, additional tests are needed for the GenRead system before such a project can be started.

In spite of many advances of NAATs, the major limitation of all molecular assays in clinical bacteriology is the lack of simultaneous provision of comprehensive antimicrobial susceptibility pattern. However, the number of resistance markers that can be targeted by molecular techniques is increasing rapidly and in the near future many bacterial resistance genes may be routinely detectable. Along with this progress, molecular assays will eventually replace the current conventional methods in the detection of microbes in clinical specimens.

Continuous advances in the next-generation sequencing (NGS) methods have the strongest potential to transform diagnostics in clinical microbiological laboratories in the next few years. With NGS, organisms can already be accurately identified and strain-typed for outbreak or transmission analyses (287-289). As NGS technology becomes more amenable to use in the clinical laboratory and the associated cost decreases with the small-scale sequencing machines and advancement of the bioinformatics pipeline, there is likely to be a concomitant increase in use of sequencing in prediction of antimicrobial resistance.

In conclusion, it is important to familiarize with the new NAATs in sufficient depth to enable them to be properly implemented in clinical diagnostics. Acquiring and utilizing an optimal set of modern NAATs requires microbiologists a know-how to compare and evaluate different solutions that best fit the laboratory or hospital environment.

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