Molecular detection to improve surveillance of multi-resistant bacteria

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1

GENERAL INTRODUCTION

Introduction

Antimicrobial resistance is emerging worldwide and considered one of the major threats in healthcare, since it is associated with increased morbidity, mortality and higher costs of treating infectious diseases.¹ The increasing resistance to all classes of antibiotics, sometimes resulting in multidrug or even pandrug resistance of some bacterial strains or species, is due to the exponential use since their introduction. This is not only caused by the increasing use for the treatment of bacterial infections in man, but also by the increasing use in the farming industry. Here, antibiotics were included in the food of pigs, chicken and several other animals, primarily for disease prevention, growth promotion and treatment of enteric diseases.²⁻⁴

Since the discovery of penicillin and other new antimicrobial compounds (figure 1), studies have clearly shown their importance for the treatment of all kinds of infections. It has been reported that the rate of mortality could be reduced by 10% up to 75% in skin infections, community pneumonia, nosocomial pneumonia, bacterial meningitis and bacterial endocarditis, comparing the use of effective antibiotics to the clinical outcome of these infections in the pre-antibiotic era.⁵ This clearly demonstrates the need for effective antibiotics. However, the fact that there is a void in development of new antibiotics since 1987 is very worrisome and gives bacteria the opportunity to further develop resistance to current antibiotics.⁶

This increasing antimicrobial resistance has major consequences. First, for the individual patient, resistance is responsible for difficulties in optimal treatment of infections caused by microorganisms that are resistant to antibiotics, possibly resulting in increased morbidity and mortality. Second, increasing resistance is observed in hospitals, followed by nosocomial transmission and outbreaks.⁷ This problem of increasing antimicrobial resistance is continuously challenging infection control and hygiene procedures in hospitals, that have been developed to prevent transmission of resistant bacteria leading to hospital acquired outbreaks. Finally, antimicrobial resistance has become a global problem. Mainly due to the increase of travelling, worldwide dissemination of antimicrobial resistance is seen. As a consequence, resistance can rapidly emerge everywhere in the world.

Mechanisms of resistance

Antimicrobial resistance of bacteria can be mediated by various mechanisms, in which one or multiple mechanisms in parallel can be responsible for resistance. These mechanisms can be obtained from other bacteria or the environment. The most important mechanisms of resistance in bacteria are i) efflux of the antimicrobial compounds from the cell, ii) decreased or no affinity to the target by alteration of the antibiotic binding site and iii) hydrolysis of the antibiotic by enzymes.

The efflux mechanism is mediated by so-called efflux pumps that eliminate endogenous metabolites that are lethal to the bacteria, either Gram-positive or Gram-negative. The efflux pumps recognize the antibiotics as 'accidental substrates' and extrude the antibiotics by use of different proteins.⁸

Traditional efflux pumps excrete only one drug or one class of drugs, while multidrug efflux pumps can pump out a wide range of compounds. The combination of such efflux pumps together with the narrow porin channels that attribute to an outer membrane barrier, is mostly responsible for intrinsic resistance of bacteria.⁹ Moreover, the presence of certain additional (efflux) genes has been associated with increased resistance to the antibiotics.^{8,10}

FIGURE 1 Dates of discovery of distinct classes of antibiotics. Dates indicate initial discovery or patent (adapted from Silver⁶, with permission of ASM)

Resistance to antibiotics caused by target alteration is a result of (a) mutation(s) usually accumulating in genes that have shown to be important for the replication of the strain. Resistance to beta-lactam antibiotics such as penicillin and amoxicillin can be the result of target alteration. Penicillin-binding-proteins (PBPs) are enzymes that catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell-wall. Normally, beta-lactam antibiotics will bind to the active site of the PBPs, resulting in inactive enzymes followed by the death of the bacteria. However, alteration of the PBPs can occur, with subsequently lower affinity for the binding of beta-lactam antibiotics resulting in decreased susceptibility or even resistance.¹¹

A well-known resistance that is caused by PBP alteration is methicillin resistance in the clinically important *Staphylococcus aureus*. Several studies have confirmed that MRSA strains produce an additional PBP, known as PBP2a (or PBP2') and have a low affinity for methicillin, resulting in high MICs.^{12,13} This PBP2a is mediated by the presence of the *mecA* gene,^{14,15} that is carried by a (presumed to be mobile) genetic element named as staphylococcal cassette chromosome *mec* (SCC*mec*).¹⁶

Although PBP alteration is frequently found to be the cause of resistance to beta-lactam antibiotics in staphylococci and streptococci, in general, the most common cause of resistance to these antimicrobial agents are beta-lactamase enzymes that hydrolyze the antibiotic. Beta-lactamases have been classified in four major classes based on amino acid homology, Ambler class A to D.¹⁷ As shown in figure 2, most beta-lactamase enzymes (Ambler classes A, C and D) use a free hydroxyl molecule that is linked to a serine residue at the active site of the enzyme to disrupt the active site of the antibiotic: the beta-lactamring. Beta-lactamase enzymes of the molecular class B (metallo-beta-lactamases, MBLs) utilize zinc ions to hydrolyze the beta-lactamring.^{18,19} Beta-lactamases can be both chromosomal- or plasmid-mediated, in which plasmid-mediated beta-lactamases are of special interest because of their ability to spread between bacteria.^{17,20} The initial, wild-type beta-lactamases are TEM-1, TEM-2, SHV-1, that both belong to Ambler class A,^{21,22} of which TEM-1 is the most widespread and responsible for up to 94% of ampicillin-resistance in *Escherichia coli* isolates.¹⁹ In order to treat infections caused by isolates that contain wild-type beta-lactamases, second and third generations of beta-lactam antibiotics were developed. Unfortunately, together with the introduction of the new beta-lactam antibiotics, resistance emerged rapidly as a result of new beta-lactamases showing increased levels of resistance to beta-lactam antibiotics.

FIGURE 2 Schematic overview of the hydrolysis of the intact beta-lactam ring (A) by the different groups of beta-lactamases (adapted from <http://what-when-how.com/molecular-biology/beta-lactamases-molecular-biology>)

These new beta-lactamases, called extended-spectrum beta-lactamases (ESBLs) are derivatives of the wild-type TEM-1 and SHV-1 beta-lactamases in which single mutations can be related to resistance in third-generation cephalosporins.^{23,24} The first ESBL, reported in 1985, was SHV-2 found in a single strain of *Klebsiella ozaenae* isolated in Germany. Since then, more than 210 TEM-variants and more than 180 SHV-variants are identified as ESBL producer. These sequences, all showing one or multiple mutations in the parent genes, are published online at the 'Lahey database' (<http://www.lahey.org/studies/>).

However, not only TEM- and SHV-variants show resistance to third-generation beta-lactam antibiotics. In the late 1980s and the beginning of the 1990s, a non-TEM, non-SHV ESBL was detected, responsible for resistance to cefotaxime. This ESBL, detected in different parts of the world, was in several reports designated as either FEC-1, CTX-M1 or Toho-1. Sequencing these genes revealed high similarity, subsequently leading to renaming the Ambler class A enzymes to CTX-M1, CTX-M2 or CTX-M3. Since then, the CTX-M enzymes are a rapidly growing family of ESBLs worldwide, in a wide range of clinical bacteria, especially in *Enterobacteriaceae*.²⁵ In contrast to TEM- and SHV-ESBL, in which point-mutations in parent enzymes are responsible for ESBL, the origin of CTX-M enzymes has been traced back to chromosomally encoded enzymes of environmental *Kluyvera* spp. According to the Lahey database, over 150 different genes of CTX-M have been identified, which can be divided on the basis of amino-acid changes.²⁶ As shown in figure 3, the CTX-M ESBLs comprise of one minor and five major groups (i.e. CTX-M1, CTX-M2, CTX-M8, CTX-M9, CTX-M25 and CTX-M45). The major groups are each consisting of a subgroup of several plasmid-mediated enzymes that share >96% identity.²⁷

FIGURE 3 Overview of the different groups of CTX-M ESBLs. Filled triangles at the end of each branch indicate the presence of minor allelic variants within the specific group (adapted from Rossolini *et al.*²⁷, with permission of John Wiley and sons)

Although most identified ESBLs belong to either the TEM-, SHV- or CTX-M group, several other ESBLs have been described. Additional clinically relevant types of ESBLs are PER, VEB,

GES or OXA and, albeit very rare, CME, SFO, TLA, BES and BEL.²⁸ As shown in table 1, besides OXA that belongs to Ambler class D, these ESBLs all belong to Ambler class A, but do not have much homology with TEM-, SHV- and CTX-M ESBLs.²⁹ The primary detected OXA-enzymes were capable of hydrolyzing oxacillin and cloxacillin only. The first variant that was able to hydrolyze third generation cephalosporins by its extend-spectrum beta-lactamase was OXA-11.³⁰ Today, the OXA-type enzymes is a growing family of ESBLs mainly found in *Pseudomonas aeruginosa*.

TABLE 1 Ambler classes and their beta-lactamase enzymes with in brackets their location. In bold are the mostly identified beta-lactamase genes

Ambler class	ESBL (plasmid)	ESBL (chromosomal)	Carbapenemase (plasmid)	Carbapenemase (chromosomal)
A	TEM, SHV, CTX-M , PER, VEB, CME-1, SFO, GES-1, BES, TLA	BEL	IMI-2, GES-2, KPC	SME, NMC-A, IMI-1
B			NDM, VIM, IMP , GIM, SIM, DIM, SPM	BCII, CphA, L1
D	OXA-types		OXA-23, OXA-48- types	OXA-23

Since third-generation cephalosporin antibiotics are no longer effective when an ESBL producer is identified, the antibiotic of choice is generally coming from the group of carbapenems, a highly effective antibiotic for the treatment of infections due to Gram-negative nosocomial pathogens. However, as with the ESBLs, shortly after the introduction of the carbapenem antibiotics, resistance was observed. This resistance is mediated by a variety of carbapenemase enzymes, belonging to Ambler class A, B and D.

Ambler class A beta-lactamases comprise the enzymes SME, NMC-A, IMI, GES and KPC (table 1).^{30,31} Besides GES, all genes encoding carbapenemases were initially found to hydrolyze carbapenem antibiotics. Although GES-1 possesses cysteine residues as found in carbapenem-hydrolyzing class A beta-lactamases NMC-A, IMI-1 and SME-1, no significant hydrolysis of the carbapenem antibiotic imipenem was proven. Therefore, GES-1 was designated as ESBL.³² Not long after the first report of GES-1, Poirel *et al.* described the beta-lactamase GES-2, that differed by one amino acid change from GES-1 and showed to be able to hydrolyze imipenem in *P. aeruginosa*.³³

Metallo-beta-lactamases (MBLs) belong to Ambler class B and include BCII, CphA, L1, NDM, VIM, IMP, GIM, DIM, SPM and SIM-enzymes. The main characteristic of all MBLs is the hydrolysis of carbapenems by interaction of the beta-lactams with zinc ions in the active site of the enzyme (figure 2). Most of the enzymes hydrolyze cephalosporins and penicillins but lack the ability to hydrolyze aztreonam.³⁰

The first OXA-enzyme that was able to hydrolyze carbapenems, was a novel beta-lactamase

found in an *A. baumannii* isolate (ARI-1: *Acinetobacter* resistant to imipenem). This enzyme, later designated as OXA-23, was found to be resistant to imipenem, penicillins and all classes of cephalosporins.^{30,34} In 2001, a novel plasmid-mediated oxacillinase was detected in a *K. pneumoniae* isolated in a hospital in Istanbul, Turkey. This oxacillinase, OXA-48, showed strong carbapenem-hydrolyzing activity and was resistant to all available beta-lactams.³⁵ A high number (>300) of unique OXA-sequences have been identified and presented in the Lahey database. Of the OXA-sequences, the OXA-carbapenemases can be divided in several subgroups, based on amino acid homologies.^{30,36}

Transmission of resistance

Most resistance mechanisms are regulated by a wide variety of resistance-genes, that can be located chromosomally or on mobile genetic elements, such as transposons or plasmids. Although all genes encoding resistance to antibiotics are responsible for decreased susceptibility, genes that are located on mobile elements have the capacity for horizontal gene transfer and dissemination of the resistance-genes across different bacterial strains and species.^{37,38}

The *mecA* gene, responsible for MRSA, is located on a genetic element (pathogenicity island) that is designated staphylococcal cassette chromosome *mec* (*SCCmec*).¹⁶ The emergence of methicillin resistance is due to the insertion of this *SCCmec* into the chromosome (flanking the *orfX* region) of susceptible strains. Many variations of *SCCmec* elements have been described, based on the composition of for instance the *ccr* gene complex, *mec* gene complex and/or J regions.³⁹ Over time and under selective pressures of for instance antibiotics, the cassettes have diverged by accepting different transposons, plasmids and other integrating elements to create multi-resistance MRSA strains.⁴⁰ Dissemination of MRSA occurs by transmission of the microorganism itself. Several reports have shown that MRSA may spread to close contacts within for instance the same household or even by contamination of environment such as the telephone or television remote control.^{41,42}

Plasmid mediated transmission is the most common mechanism of horizontal gene transfer, transmitting genes from one strain to another. When a beta-lactamase mediating gene is located on such plasmid, this gene can be transferred from one strain to another, expressing the same beta-lactamase in this isolate.^{43,44} This phenomenon has shown to be responsible for worldwide dissemination of resistance genes, as for instance CTX-M ESBL.²⁵

In the past decade, animals have become an important vector and source of resistant bacteria. In 2004, the group of Voss *et al.* unexpectedly identified MRSA from a patient, with no history of MRSA suspicion (e.g. travelling, admission to a foreign hospital).⁴⁵ In this study, the first transmission of MRSA between pigs and human was demonstrated. This finding was confirmed by other groups, all showing high prevalence of MRSA in pigs.^{46–48} This MRSA, belonging to sequence type ST398, was also found in veal calves, showing the same possibility of transmission between animal and human and is known as livestock-associated MRSA (LA-MRSA).⁴⁹ In 2011, a new variant of MRSA was reported. This MRSA, found in bovine cattle,

showed to harbor a novel homologue of the *mecA* gene (*mecA*_{LGA251}).⁵⁰ Since the gene of this novel homologue was only 70% identical to the sequence of *mecA*, existing detection methods were not able to correctly identify this MRSA. Subsequently, this novel *mecA* homologue was renamed and should now be reported as *mecC*.⁵¹

Animals have also shown to be colonized with ESBL producing isolates. Presence of ESBL, as detected in companion animals, may be a reservoir of spread of the resistance genes to the community.^{52,53} Furthermore, since ESBL-encoding genes have been found in chicken and pigs, both part of the food chain, showing similarity with the ESBL variants present in human, further dissemination of these resistance determinants may be a risk.^{54,55}

However, the most important source for worldwide dissemination of both MRSA, ESBL and carbapenemase producing isolates is international travel, because of for instance (medical) tourism or human migration. Although MRSA mostly is obtained by hospitalization in foreign hospitals, for ESBL or carbapenemases, travelling to countries with endemic situations has proven to be a major factor for the introduction of multiresistant *Enterobacteriaceae* into the community.^{56–58} This global spread of MRSA, ESBL and carbapenemase genes is a major threat for both the individual patient and for healthcare-institutions.

Epidemiology

MRSA

The first reports of both single cases and outbreaks of MRSA appeared not long following the first report on the detection of MRSA.^{62,63} The first hospital outbreak of MRSA has even been described in the same year as the first finding of MRSA and consisted of 71 MRSA-isolates from 37 individuals.⁶³

After the introduction, a new raise in the prevalence of MRSA was determined in the late 1970s and early 1980s, associated with the introduction of “new-MRSA” or “modern-MRSA”, showing co-resistance to antimicrobials such as tetracycline, gentamicin and clindamycin.^{64,65} In the same time, transfer of MRSA between countries became an important factor for the introduction of MRSA in hospitals.⁶⁶ As a consequence, this multi-resistant MRSA was emergently detected worldwide.^{65,67}

Up to the mid 1990s, most MRSA cases could directly be linked to hospital or other health-care institutions. However, starting in the mid 1990s, increasing reports on MRSA with no direct linkage to any health-care facility appeared.^{68,69} These MRSA isolates, called community-associated MRSA (CA-MRSA), differed from the hospital-associated MRSA (HA-MRSA) by carrying a smaller *SCCmec* element and have predominantly been isolated from skin and soft tissue infections associated with the presence of the toxin-producing gene encoding Panton-Valentine Leukocidine (PVL).⁷⁰ Following the introduction of CA-MRSA, the prevalence rapidly increased worldwide with various MRSA clones that have spread between the community and the hospitals.^{71–73} Chua *et al.* showed several dominant MRSA clones per country or region,

for instance a huge clone of ST8, *SCCmec* type IV (called USA300) in the USA and a major “European-clone” (ST80, *SCCmec* type IV), while the clones in Australia vary from ST1, ST30 to ST93 (all *SCCmec* type IV) throughout the country.⁷⁴

Next to CA-MRSA, the introduction of ST398 (LA-MRSA) has shown to be a threat for healthcare and hospital outbreaks. Although LA-MRSA accounted for only a small proportion of the total of MRSA isolates from humans, high rates of MRSA carriage by human in contact with pigs have been detected.⁷⁵ Initially, it was thought that LA-MRSA could only be found in persons exposed to transmission from pigs or veal-calves. In 2010, however, a case report on a ventilator-associated pneumoniae caused by LA-MRSA was published. This case, in which the patient did not have any exposure to zoonotic clonal lineages or history with any other MRSA risk factors, implies possible spread of LA-MRSA within the community.⁷⁶

FIGURE 4 MRSA prevalence in European countries according to the database of EARS-net (2011)

Since there is no worldwide harmonization in the determination of the prevalence of MRSA, comparison of different regions globally is not feasible. Nevertheless, some countries or even continents have established surveillance programs to get an indication on the prevalence of MRSA. The outcomes of such surveillance programs demonstrate that the prevalence of MRSA all over the world is >20%, except for the northern countries in Europe. The latter is illustrated by an interactive database of the European Antimicrobial Resistance Surveillance Network (EARS-net, organized by the ECDC), that shows the prevalence of methicillin resistance in *S. aureus* isolates coming from blood cultures and cerebrospinal fluid, from European countries attending the surveillance program. In 2011, the prevalence of MRSA in Europe ranges from <2% up to 54.6%, with an overall low prevalence in the northern countries and a high prevalence in the southern countries (figure 4). In the rest of the world, as determined by regional working groups on antimicrobial resistance, the prevalence of MRSA is 55.5% (2008, 48.6% - 68.2%), 23% (2007-2011), 52.5% (2004-2007, 16.5% - 81.0%), 33.6% (2009, 27.3% - 41.4%) and 40% (2003-2008, 32.7% - 49.7%) for the USA, Canada, Asia, Australia and Latin America respectively.^{73,77-80} For Africa, Falagas *et al.* reviewed the data from publications and found the prevalence of MRSA between 25% - 50%.⁸¹

ESBL

During the 1990s, reports of ESBLs mostly described resistance mediated by TEM- and SHV-ESBL genes, that are detected in a broad range of genera of *Enterobacteriaceae* and mostly *K. pneumoniae* respectively.²⁹ Although CTX-M ESBL was first detected in the late 1980s, a worldwide expansion was not seen until 1995. However, already in 1989 an explosive dissemination of *Salmonella* spp. strains with CTX-M2 ESBL was observed in Southern America, with spread between a high diversity of strains in different hospitals and even countries.²⁵

Other than TEM- and SHV-derived ESBLs, the CTX-M enzymes are not limited to nosocomial infections mainly caused by *Klebsiella* spp. Since they can spread beyond the hospital environment, the public health concern raises. Especially *E. coli*, often containing CTX-M enzymes, seems to be a true community ESBL pathogen.⁸² Studies have shown the presence of ESBL in both community patients, outpatients and healthy volunteers illustrating the widespread of ESBL-producing bacteria.⁸³

The prevalence of ESBL producers differs worldwide, with the number of ESBL producing *K. pneumoniae* and *E. coli* isolates being highest in Southern America (respectively 44.0% and 13.5%), followed by the Asia/Pacific region (respectively 22.4% and 12.0%).⁸⁴ However, not only the prevalence is region dependent, also the type of ESBL appears to be unique in a certain region or country, or even in an institute. For instance, TEM-3 is common in France, but has not been detected in the USA. SHV-12 and SHV-2a are the most common found ESBL in Korea, while SHV-5 is commonly encountered worldwide.²⁹ This holds also true for the CTX-M ESBLs as nicely shown in figure 5.

Although the most prevalent ESBL types are TEM, SHV and CTX-Ms, other ESBL types can

be of epidemiologically interest e.g. PER-ESBL in Turkey (*A. baumannii*: 60% ceftazidime-resistance).²⁹

FIGURE 5 The dissemination of CTX-M ESBLs over the world (adapted from Hawkey and Jones ¹, with permission of Oxford Journals)

Carbapenemase

From both Ambler class A,B and D, genes encoding carbapenemase enzymes have been detected located either on the bacterial chromosome or on mobile elements (plasmid). Although all resistance genes can mediate decreased susceptibility, resistance genes located on the bacterial chromosome are of less epidemiological importance since the genes lack the ability to disseminate among different bacterial strains and species. This is in contrast to resistance genes located on plasmids, that do have the capacity for horizontal gene transfer.

Klebsiella pneumoniae carbapenemase (KPC) was first reported in 2001 by Yigit *et al.* and named after the isolate in which it was first detected.⁸⁵ Amongst Ambler class A, KPCs are clinically the most common enzymes, while IMI-2 and GES-2 have only rarely been described.

Although KPC is named after the isolate in which it was first detected, they have now been described in many other species of *Enterobacteriaceae* and *P. aeruginosa*.³¹ Even though KPC-1 was first detected in the USA, reports describing different variants of KPC that were detected worldwide began to appear rapidly. Today, KPC is most prevalent detected (up to endemic situations) in eastern-USA, parts of Southern-America and around the Mediterranean sea

(Greece, Italy, Israel).^{86,87} An analysis by MLST typing of 16 different *K. pneumoniae* isolates coming from 6 different countries, USA, Greece, Sweden, Colombia, Brazil and Israel, showed 10 isolates with the same clone (sequence type (ST)258) or single- or double locus variants coming from all countries except Colombia.⁸⁸ The finding of this single clone in isolates coming from a variety of countries indicates that such clone may have contributed to the global spread of the KPC genes.

Of the MBL carbapenemases, GIM-1, DIM-1, and SIM-1 and SPM-1 have only been reported once or sporadically. In contrast, Ambler class B carbapenemases VIM, IMP and NDM clearly show worldwide dissemination.^{30,89} Both VIM- and IMP carbapenemases originate from a *P. aeruginosa* strain, respectively in Italy and Japan^{90,91} and have shown global spread, with an overall trend of these two MBLs moving beyond *P. aeruginosa* and *Enterobacteriaceae*.³⁰ Today, endemic spread of VIM carbapenemase (mostly VIM-2⁹²) is seen in Greece, while interhospital spread is shown in Spain and Italy.^{86,87} IMP carbapenemase continues to persist in Japan and Taiwan while nosocomial outbreaks are described in Canada (IMP-7, *P. aeruginosa*), Australia (IMP-4, mainly *S. marcescens*) and Italy (IMP-13, *P. aeruginosa*).^{93–95} New-Delhi Metallo-beta-lactamase (NDM) mediated resistance was first identified in 2008,⁹⁶ yet global spread of this MBL has already occurred.⁸⁷ Cases have been reported worldwide, of which some infections with NDM producers could be traced back to India, Pakistan, Bangladesh, the Balkan states or the Middle East, all countries with a high prevalence (up to endemic situation) of this MBL. NDM has even been identified in *E. coli* ST131 as a source of community-acquired infection. This is of particular concern, since ST131 is known to mobilize the ESBL CTX-M15 efficiently worldwide.⁸⁹

The first class D beta-lactamase that was able to hydrolyze carbapenems is OXA-23, that was found to be resistant to imipenem, penicillins and all classes of cephalosporins.^{30,34} Although OXA-23 is mostly chromosomal encoded, it has sporadically been detected on a plasmid as well.³⁶ After its initial identification in Turkey, the emerging plasmid-mediated OXA-48 has disseminated within that country, where nosocomial outbreaks occurred.⁹⁷ Moreover, the carbapenemase was also disseminating further in the Middle-East, North Africa and finally whole Europe.^{98–101} Although the prevalence of OXA-48 is rapidly increasing in Northern Africa and Europe, cases of OXA-48-type carbapenemases are sporadically reported in other parts of the world. However, since OXA-48-type producers are likely the most difficult carbapenemase producers to be identified, because of low MICs and the fact that not all OXA-48 producers co-produce ESBL, their true prevalence might be underestimated.⁸⁷

Overall, although the prevalence and type of carbapenemases differ, an increase in detection of carbapenemase producing isolates is seen worldwide. Especially in the Indian subcontinent, of which prevalence data suggest that NDM-1 is present in hospitals at a rate of 2-8%. However, this may only be the tip of the iceberg, since it is almost certain a community-associated resistance.⁸⁹ In Europe, carbapenem resistance is increasing to alarming rates. As illustrated in figure 6, a massive increase of carbapenem resistance is observed in *K. pneumoniae* isolates coming from blood cultures and cerebrospinal fluid. Comparing the results of the EARS-net database (2006 and 2011), a significant increase in carbapenem resistance is seen in Greece, Italy and Cyprus.

FIGURE 6 Proportion of carbapenem resistant *Klebsiella pneumoniae* isolates coming from blood cultures and cerebrospinal fluid in participating countries, 2011 (EARS-net)

Screening and detection

In the Netherlands, the working party for infection control (WIP) has developed guidelines to prevent introduction and possible spread of highly resistant microorganisms (HRMO) such as MRSA, ESBL and CPE.^{102,103} In these guidelines, the WIP describes recommendations in order to prevent spread of HRMOs, consisting of for instance isolation measurements, contact tracing and surveillance of HRMO. Moreover, for the latter, the guidelines describe when surveillance for HRMO should be performed and what patients should be included.

For a successful policy regarding the HRMOs, not only the recommendations as described by the WIP are important, also the correct and rapid detection of these microorganisms is of high importance in order to install adequate consequential measures. New methods such as selective media or PCR assays have been developed in order to screen for the presence of HRMOs in patients of risk.

Phenotypic detection

A wide range of different selective media have been developed for the rapid detection of HRMOs. The first selective medium has been developed in the 1980s and was able to screen for the presence of MRSA.^{104,105} Currently, selective media are also available for the detection of ESBL and CPE. In most selective media, antibiotic supplements and chromogenic substrates are included for direct identification of the resistant microorganism and differentiation between different species respectively.^{106–108} However, the performance of selective media differ largely, due to a wide variety of reasons.^{106,108,109} Therefore, isolates suspected to be either MRSA, ESBL or CPE should always be confirmed. Here, usually the first step is to determine the susceptibility pattern, mostly done by disk diffusion, using several disks impregnated with antibiotics, or automated systems such as Vitek2 (BioMérieux), Phoenix (BD diagnostics) or MicroScan (Siemens Healthcare diagnostics).

Subsequent confirmation of MRSA is based on the detection of the PBP2a by a latex agglutination test. This assay contains latex particles sensitized with monoclonal antibodies against PBP2a from *S. aureus* and has high sensitivity and specificity, yielding results within 20 minutes.¹¹⁰

Confirmation of ESBL- and carbapenemase producers is performed by so-called inhibition assays and interpretation is rather complicated. Inhibition assays use the presence of a beta-lactamase inhibitor (clavulanic acid/sulbactam for ESBL or boronic acid/cloxacillin for CPE) next to a beta-lactam or carbapenem antibiotic. The test is considered positive when the zone of inhibition of the beta-lactam or carbapenem antibiotic together with the beta-lactamase inhibitor is enhanced, because of synergy, compared to the inhibition zone of the disc with only the beta-lactam/carbapenem antibiotic. For the confirmation of CPE, the Modified Hodge Test (MHT), based on the *in vivo* hydrolyzation of carbapenem antibiotics, can also be used.¹¹¹ However, all methods have shown problems in the correct identification of ESBL or CPE. Since overproduction of other beta-lactamase enzymes can cause the MIC of beta-lactam antibiotics to rise, false positive results may occur.^{29,87,112}

To overcome the issue of sensitivity and specificity, new methods have been developed, especially for the detection of CPE. A relatively new method is the detection of carbapenemase using Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, that is based on the analysis of the degradation of meropenem. Although this method is time-consuming and laborious, it did show sensitivity and specificity of 96.7% and 97.9% respectively.¹¹³ Finally, Nordmann and colleagues introduced the Carba NP test. This test has shown 100% sensitivity and specificity, making it an easy and rapid method to detect the presence of a carbapenemase enzyme.¹¹⁴ Moreover, an extended version of the test has been developed that can discriminate between the different types of carbapenemases (classes A, B and D).¹¹⁵

Molecular detection

Many different phenotypic methods to detect and confirm the presence of MRSA, ESBL and CPE have been developed and evaluated. Some demonstrate good results, but many show lack of sensitivity and specificity. In these cases, confirmation by molecular methods is obligatory, by use of for instance polymerase chain reactions (PCR) with or without subsequent sequencing or real-time PCR assays. In both options, oligonucleotide sequences (primers and probes) are developed targeting the gene of interest. Using a thermal profile and Taq polymerase, these primers and probes will bind to the targeted gene and multiply the specific gene. Eventually, when the gene is present, it can be detected by sequencing or a labeled probe (real-time PCR). (figure 7)

After the introduction of polymerase chain reactions (PCR), direct detection of the *mecA* gene was possible.^{116,117} Confirmation of the presence of the *mecA* gene was determined to be the gold standard, resulting in many new (real-time) PCR assays to detect the *mecA* gene. When a *mecA* PCR in combination with a PCR targeting a *S. aureus* specific-gene, such as the SA442- or the *nuc*-gene, is applied on a strain, direct detection of MRSA is possible.^{118,119}

FIGURE 7 Illustration of the principle of real-time PCR (picture adapted from <http://technologyinscience.blogspot.nl>)

Genotypic confirmation of ESBLs and CPE can be performed by different molecular methods such as (multiplex) PCR, PCR with subsequent sequencing or electrophoresis, real-time PCR with or without melt-curve analysis^{120–123} or DNA micro-array analysis.^{124–127} Although many assays are able to detect only one group of beta-lactamase, such as CTX-M or KPC, it is also possible to identify multiple beta-lactamase genes by use of multiplex assays. As with all molecular methods, the used assays will only detect the genes for which they have been developed. Nonetheless, next to the fact that molecular assays are rapid and easy to interpret, the possibility of molecular methods to directly differentiate between different genes, is an important tool from an epidemiological point of view.

In order to increase the speed of detecting MRSA, ESBL and CPE, researchers aimed to develop molecular assays that can be applied directly on clinical samples. For MRSA, combining PCRs targeting the *mecA*-gene and a *S. aureus* specific gene has shown to be a helpful tool in confirming the presence of methicillin resistance in a *S. aureus* isolate. However, using this combination of PCRs directly on a clinical sample containing a mixture of staphylococci, a false positive signal can occur because of the combination of a methicillin susceptible *S. aureus* (MSSA) and methicillin resistant coagulase negative staphylococci (MRCNS). To overcome this problem, Huletsky *et al.* developed a real-time PCR assay targeting the *SCCmec*-cassette and *S. aureus* specific *orfX* gene that is useful for the detection of MRSA directly from non-sterile specimens.¹²⁸ Although false-positive results might occur because of the presence of the *SCCmec* cassette without a *mecA*-gene (4.6% of the tested MSSA isolates), 98.7% of the MRSA isolates were detected correctly.¹²⁸ The principle of this assay is used for several commercially available applications such as the IDI-MRSA assay (BD diagnostics) or the GeneXpert MRSA assay (Cepheid).^{129,130} A disadvantage of the existing assays based on the known *SCCmec* cassettes, is the lack of detection of new variants (for instance some of the LA-MRSA) that do appear occasionally.⁵⁰ Consequently, these assays should be updated in order to detect all possible *SCCmec* cassettes. Nonetheless, using these assays as a part of the search and destroy policy has shown to be a valuable tool for rapid detection of MRSA and a major step in reducing the number of isolation days.^{131,132}

Although several molecular assays have been developed, able to detect a wide range of genes mediating ESBL, these assays have mostly been evaluated on clinical isolates only. Few assays have been described able to detect ESBL genes directly from clinical samples. So far, only assays that can detect CTX-M genes directly from urine or positive blood cultures have been described.^{133,134} No assay has been evaluated detecting ESBL genes directly from rectal swabs, that are mostly obtained to screen for the presence of HRMO.

In contrast to ESBL, a few real-time PCR assays have been developed able to detect specific CPE genes directly in DNA isolated from rectal swabs. These assays have shown a high sensitivity and specificity in detection of either *bla*_{KPC}-genes or *bla*_{NDM}-genes.^{135–137} However, by our knowledge, no assays have yet been developed capable of detecting all epidemiologically important carbapenemase genes (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM}, *bla*_{OXA-48}) directly from surveillance samples.

Aim and outline of this thesis

Resistance to antimicrobials is emergently detected worldwide, including the intrinsic and chromosomally mediated resistance, as well as the resistances that are mediated by genes located on mobile elements. Of these, mobile elements have shown to be very important vehicles since they attribute to the rapid dissemination and global spread of genes mediating antimicrobial resistance. To prevent further dissemination of antimicrobial resistance, rapid and correct detection is necessary in order to install adequate infection control measures. As described earlier, new methods have been developed to detect the resistant strains more rapidly. The introduction of selective media has already shown to be important and reduced the time to identify MRSA with 12 to 16 hours, compared to routine isolation and susceptibility techniques.¹⁰⁴ A further decrease in time to result was seen when molecular testing was introduced. The same holds true for multiresistant Gram-negative bacteria. For instance, when molecular testing for the presence of KPC was compared with several selective media, a significant decrease in turn-around-time was seen in favor of direct molecular testing (24 hours versus 60-75 hours). Furthermore, the performance of the molecular methods showed to be superior over the selective media.^{135,138,139}

Obviously, the introduction of new methods has shown to be responsible for more rapid detection of antimicrobial resistance. This has positive consequences for the individual patient, infection control programs and possibly the global problem of transmission of antimicrobial resistance. For the individual patient more rapid diagnostics will result in a rapid change to adequate treatment when necessary. For infection control programs, rapid detection of (multi-) resistant microorganisms enables to rapidly install infection control measurements preventing further spread of these microorganisms. This will not only save money but also reduce unnecessary isolation days, which is convenient for both the patient and nursing personnel. Finally, rapid recognition of antimicrobial resistance on a large scale may even have a positive effect on the reduction of the spread of resistant strains at a global level. In the end, this strategy could delay the global spread of antimicrobial resistance, gaining time to develop new antimicrobial agents.

Remarkably enough, in laboratories of clinical microbiology, rapid molecular detection of resistance is still anything but the standard. For instance, in the Dutch guideline on the detection of HRMO (2012), as composed Dutch by the society for medical microbiology (NVMM), laboratory detection of HRMOs still has to rely on the initial use of selective media, with subsequent (partial molecular) confirmation.¹⁴⁰

The main objective of this thesis was not only to improve laboratory detection of HRMOs by introducing molecular assays able to trace all kind of resistant genes, but also to show the clinical importance of these molecular assays for the rapid and reliable detection of resistance genes.

The first part of this thesis describes molecular methods to confirm the presence of ESBL genes

in putative producers. In **chapter 2**, we introduce a novel more rapid (molecular) method for the confirmation of ESBL. This method, the ligation-mediated real-time PCR assay, is evaluated and the performance of the assay is compared with the recommended combination disc test.

The added value of the DNA microarray, recommended to be an ESBL confirmatory method by the NVMM guideline, is the characterization of ESBL genes. Using this aspect of the DNA microarray, we were able to describe the distribution of the most prevalent ESBL genes in our region and to compare it with other studies in the Netherlands. (**chapter 3**)

The second part of the thesis describes the clinical application of broth enrichment with subsequent molecular testing. In **chapter 4**, the results of a molecular screening approach for MRSA over a 2-year period, based on the combination of two different real-time PCR assays, is reported. In **chapter 5**, we describe the introduction of a new screening method to detect carriage of CPE in patients using overnight broth-enrichment and subsequent detection using a ligation-mediated real-time PCR assay that is able to detect the most prevalent CPE genes.

Direct molecular detection of CPE is the final part of this thesis. For the detection of CPE, directly from rectal swabs or stool, several real-time PCR assay have been developed and evaluated. However, these assays were able to detect only one group of carbapenemase genes. In **chapter 6**, the performance of a new real-time PCR assay for rapid detection and differentiation of KPC, VIM/NDM, and OXA-48 carbapenemases is evaluated. The performance of this assay, developed for direct detection on surveillance swabs, is compared with the performance of selective media (as recommended by the NVMM guideline).

Finally, in **chapter 7**, the preceding chapters are summarized and discussed. In addition, a future perspective is given, with focus on other important multi-resistant microorganisms.

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2

RAPID MOLECULAR DETECTION OF EXTENDED-SPECTRUM BETA-LACTAMASE GENE VARIANTS WITH A NOVEL LIGATION- MEDIATED REAL-TIME PCR

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Abstract

Extended-spectrum beta-lactamases (ESBLs) are emerging worldwide, making rapid and adequate ESBL detection crucial for infection control measures as well as for the choice of correct antimicrobial therapy. The aim of this study was to compare the performance of a novel rapid ligation-mediated real-time PCR (LM-PCR) with a combination disc test (CDT). In total, 172 prospective putative ESBL-positive *Enterobacteriaceae* isolates from clinical specimens based on Vitek2 results were included in this study and tested with the phenotypic CDT and the LM-PCR. Positive ESBL results were obtained in 100 and 95 isolates using CDT and LM-PCR, respectively. The sensitivity, specificity, negative predictive value and positive predictive value of the LM-PCR were 99.0%, 92.2%, 98.6% and 94.0%, respectively, compared with the CDT. The LM-PCR technique provides an important reduction in turnaround time (~4.5 h versus overnight incubation using CDT) for ESBL confirmation. As a consequence, all ESBL results are available within the same day, making this assay an important tool for rapid and accurate ESBL detection.

Introduction

Extended-spectrum beta-lactamases (ESBLs) are plasmid-encoded enzymes, found frequently in *Enterobacteriaceae*, which hydrolyze third-generation cephalosporins, penicillins and monobactams. ESBLs are emerging both in the community and in hospitals and have increasingly been described worldwide since their introduction.^{1–3} The most frequently detected and clinically important ESBLs belong to the TEM, SHV and CTX-M families.⁴

Correct detection of ESBL-producing organisms is complex. Several phenotypic detection methods have problems discriminating between ESBLs and other mechanisms of resistance to beta-lactams, leading to false-positive results.⁵ DNA-based ESBL assays, on the other hand, are complex, as over 300 different ESBL genes have been described, making it almost impossible to detect all ESBLs.^{6,7} Moreover, detection is often associated with amino acid substitutions, resulting in high variations in different ESBL types.^{6,8} However, because of the emergence of ESBLs, rapid and adequate detection of the most common ESBLs is crucial for infection control measures as well as for the choice of correct antimicrobial therapy.^{9,10} Several DNA-based ESBL assays that might meet these demands have already been evaluated, comprising standard PCR^{11,12} and/or microarray analysis.^{4,8,13}

A novel ligation-mediated real-time PCR (LM-PCR) has been developed by Check-Points and is able to detect the most prevalent ESBLs (TEM, SHV and CTX-M) using a ligation step prior to the real-time PCR. The aim of this study was to evaluate this LM-PCR to detect these three prevalent ESBLs in *Enterobacteriaceae* and to compare this method with a conventional phenotypic confirmatory method.

Materials and methods

Test isolates

From June to October 2011, a total of 172 phenotypically identified *Enterobacteriaceae* isolates were isolated that screened positive for ESBL production according to Clinical and Laboratory Standards Institute (CLSI) guidelines¹⁴ by Vitek2 using AST cards 140 (for urine samples) and 141 (for all other materials) (BioMérieux, Marcy l'Etoile, France) at the Department of Medical Microbiology and Medical Immunology (Rijnstate, Velp, The Netherlands) and were included in this study. Isolates originated from clinical specimens, of which only the first of each different ESBL-positive *Enterobacteriaceae* species (by Vitek2) was included.

DNA extraction

For all putative ESBL-positive isolates determined by Vitek2, DNA extraction (NucliSens EasyMAG, protocol specific A with 110 µl DNA eluate; BioMérieux) was performed using 200 µl of a 0.6–0.8 McFarland suspension of pure culture, including a blank control sample. Before DNA extraction, phocine herpesvirus 1 (PhHV-1) DNA was added to each suspension at a final concentration equivalent to a standard threshold cycle (Ct) value of ~32 (±2 sd) in the real-time PCR.¹⁵

LM-PCR

The LM-PCR is based on single-nucleotide polymorphism detection, using the previously described Check-ESBL assay (Check-Points), and is able to detect the most prevalent ESBL types (CTX-M1 group, CTX-M2 group, CTX-M9 group, SHV-238S, TEM-104K and TEM-164S).^{4,8,13} Figure 1 shows a schematic presentation of the principle of LM-PCR. In short, a ligation step (non-cyclic: 3 min at 95 °C, 2 h at 65 °C, 2 min at 95 °C) was performed using 5 µl solution A, 2.5 µl LpE mix (including probes with an ESBL gene-specific part, a molecular beacon-binding site and a ZIP code for putative subsequent microarray analysis⁸) and 10 µl DNA. After the ligation step, real-time PCR was performed in a 30 µl volume containing 2x TaqMan Universal Mastermix (Applied Biosystems), RtE solution (Check-points, Wageningen, The Netherlands) containing generic primers and a fluorescently carboxyfluorescein-labelled molecular beacon, PhHV-1 primers and a fluorescently Cy5-labelled PhHV-1 TaqMan probe (Biolegio, Nijmegen, The Netherlands) together with 5 µl ligation product using the TaqMan 7500 system (Applied Biosystems).

To exclude false-positive results due to background signals (Ct values >37), a cut-off value was introduced using the internal control PhHV-1, for which the titration values are well known. The ESBL LM-PCR was considered positive when the ESBL signal was stronger than the PhHV-1 signal (ESBL Ct value < PhHV-1 Ct value) using a threshold of 0.05 and an automatic baseline (automatically correcting the background fluorescence detected), according to the manufacturers' guidelines.

FIGURE 1 Principle of LM-PCR. (a) Probe arms (cSeq-1 and cSeq-2) hybridize to the specific target sequence resulting in a double-stranded molecule with a single-stranded nick. (b) The nick is ligated by DNA ligase in the case of a perfect double-stranded match, connecting the two probe arms. (c, d) Real-time PCR is performed using generic primers (binding to sites cPrimer-1 and cPrimer-2) and a molecular beacon (MB) (d), using a generic molecular beacon-binding site (c-MB). The ZIP sequence, which is specific for each probe, can be used for microarray analysis.

Phenotypic characterization

Phenotypic ESBL detection was performed according to standard protocols with a combination disc test (CDT), as described previously.^{16,17} In short, discs containing a cephalosporin (cefotaxime, ceftazidime or cefepime) and a combination of this cephalosporin with clavulanic acid were placed on Mueller–Hinton agar inoculated with 0.5 McFarland suspension of the isolate. Isolates were considered positive if the inhibition zone, measured after overnight incubation at 37 °C, around the combination disc (cephalosporin+clavulanic acid) was at least 5 mm larger than the corresponding cephalosporin disc.

Discrepancy testing

Discrepant results between the CDT and LM-PCR were analyzed for the presence of TEM, SHV and CTX-M ESBLs using a conventional PCR and subsequent sequencing, as well as by the Check-MDR CT103 assay (Check-Points).

The conventional PCR and sequencing for molecular characterization of ESBLs were performed as described by Cohen Stuart *et al.*⁸ and were able to detect the ESBL genes TEM, SHV and CTX-M.

The Check-MDR CT103 assay has been well evaluated by Cuzon *et al.*¹⁸ and is able to detect most prevalent ESBLs (*bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}), plasmid-mediated AmpC beta-lactamases (*bla*_{CMY-2-like}, *bla*_{DHA}, *bla*_{FOX}, *bla*_{ACC-1}, *bla*_{ACT/MIR} and *bla*_{CMY-1-like/MOX}) and carbapenamases (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM} and *bla*_{OXA-48}).

Results

A total of 172 putative ESBL-producing *Enterobacteriaceae* were identified with Vitek2 and further analyzed by CDT and LM-PCR. Isolates were obtained from 163 patients, from a total of 168 clinical specimens (117 from urine, 17 from rectal swabs/feces, seven from sputum, six from positive blood cultures and 21 from miscellaneous specimens), and represented a total of ten bacterial species, most of which were *Escherichia coli* (101/172; 58.7%) (Table 1).

TABLE 1 Putative ESBL positive isolates screened by Vitek2

Isolate	No. isolates	CDT positive	LM-PCR positive	Discrepancies	PCR + sequencing*	CT103* (n)
<i>Citrobacter freundii</i>	1	0	0			
<i>Citrobacter species</i>	7	0	0			
<i>Enterobacter aerogenes</i>	6	0	0			
<i>Enterobacter cloacae</i>	19	7	5	2	- (2)	AmpC ACT/ MIR (2)
<i>Escherichia coli</i>	101	74	71	5	- (4) CTX-M-group 1 ESBL (1)	- (1) AmpC CMY-2 + WT-TEM (1) WT-TEM (2) CTX-M-group 1 ESBL (1)
<i>Klebsiella pneumoniae</i>	18	18	18			
<i>Klebsiella oxytoca</i>	1	1	1			
<i>Morganella morganii</i>	11	0	0			
<i>Proteus mirabilis</i>	7	0	0			
<i>Proteus vulgaris</i>	1	0	0			
Total	172	100	95	7		

CDT, combination disk test; LM-PCR, ligation mediated real-time PCR

- : Negative results (no ESBL, AmpC or carbapenemase-gene detected)

*: Discrepancy testing was performed using PCR and sequencing and the Check-MDR CT103

Positive ESBL results were obtained in 100 (58.1%) and 95 (55.2%) isolates using CDT and LM-PCR, respectively. No further differentiation between ESBL groups was determined because the currently tested molecular beacon was designed to detect only the generic ESBL groups (figure 1). In all LM-PCR tests, no inhibition was detected based on the correct PhHV-1 DNA real-time PCR signal (mean Ct value of PhHV-1 in the negative control ± 2 sd).

Comparing the results of CDT and LM-PCR, seven ESBL discrepancies were found, of which one isolate was detected that was CDT-negative/LM-PCR-positive. This LM-PCR positive result was confirmed by both conventional PCR with subsequent sequencing and the Check-MDR CT103 assay, detecting CTX-M group 1 ESBL and non-ESBL TEM. Of the remaining six

discrepancies (CDT-positive/LM-PCR-negative), all LM-PCR-negative results could be confirmed using conventional PCR and sequencing and the Check-MDR CT103 assay. Using the Check-MDR CT103 assay, in three of these isolates (two *Enterobacter cloacae* and one *Escherichia coli*), an AmpC gene (ACT/MIR in both *Enterobacter cloacae* isolates and CMY-2 in the *Escherichia coli* isolate) was detected, and in two *Escherichia coli* strains, a non-ESBL TEM was found; no resistance genes were detected in the remaining *Escherichia coli* (Table 1).

The sensitivity, specificity, and positive and negative predictive values of the LM-PCR compared with the CDT were 99.0%, 92.2%, 94.0% and 98.6%, respectively (Table 2).

TABLE 2 Results of the CDT and LM-PCR

Result	LM-PCR +	LM-PCR -
CDT positive	94	6
CDT negative	1	71

CDT, combination disk test; LM-PCR, ligation-mediated real-time PCR

Discussion

This study describes the first evaluation of a new LM-PCR method (Check-Points) that is able to detect all important amino acid substitutions (TEM and SHV) and genes (CTX-M) responsible for the most prevalent ESBLs. The present study compared this LM-PCR with a conventional phenotypic CDT. Of the 172 putative ESBL-producing *Enterobacteriaceae*, seven discrepancies were found when we compared the CDT and LM-PCR. Discrepancy testing of CDT-positive/LM-PCR-negative isolates confirmed all six LM-PCR-negative results. In three of the six isolates, an AmpC gene was detected (two *Enterobacter cloacae* and one *Escherichia coli* combined with a non-ESBL TEM), a beta-lactamase hyper-producer with similar patterns of resistance to extended-spectrum cephalosporins such as ESBL that is able to generate false-positive results in phenotypic ESBL tests by producing an increase in the inhibition zone with the combination cephalosporin–clavulanate disc.^{19,20}

Furthermore, two *Escherichia coli* isolates harboured a non-ESBL TEM (TEM-1), of which high expression has previously shown false-positive ESBL phenotypes in phenotypic ESBL confirmation methods.²¹ Wu *et al.* found that decreased outer-membrane permeability, due to loss of outer-membrane protein K35 (OmpK35), and the hydrolytic effect of TEM-1 can increase the MIC of cefotaxime slightly.²² The addition of clavulanic acid inhibits TEM-1 beta-lactamase production and reduces the MIC, causing augmentation of the inhibition zone diameter, resulting in a false-positive ESBL test result.^{19,21}

The remaining CDT-positive/LM-PCR-negative ESBL result (*Escherichia coli*) could not be confirmed by either conventional PCR or Check-MDR CT103 (ESBL-, AmpC- and carbapenemase-negative).

The LM-PCR is an easy-to-perform assay that should be considered positive when the Ct value of the ESBL is less than the Ct value of the PhHV-1 control and negative when the Ct value of the ESBL is greater than the Ct value of the PhHV-1 control because of signals in the real-time PCR (around cycle 37 and higher) caused by non-specific binding during the ligation step. Testing pure cultures of clinical isolates and using the well-known titration values of PhHV-1 as the cut-off value with the LM-PCR gives a clear signal to noise discrimination ratio between ESBL-specific and non-specific signals. Wrong (subjective) interpretation of the CDT on borderline results was probably the reason for the CDT-negative *Escherichia coli* that tested positive using LM-PCR, conventional PCR and Check-MDR CT103. Queenan *et al.* found false-negative results in phenotypic ESBL screenings using a lower range of standard inoculum, including the *Klebsiella pneumoniae* ATCC700603 SHV ESBL control strain, which could also explain the phenotypic-negative, gene-positive *Escherichia coli*.²³

The LM-PCR and the methods for discrepancy testing (PCR followed by sequencing and the Check-MDR CT103 assay) detect only the prevalent ESBL genes TEM, SHV and CTX-M. Therefore, it could not be excluded that the few LM-PCR-negative/CDT-positive ESBLs were the result of rarer ESBL genes (e.g. PER, VEB or GES). Our evaluation concentrated on detection of the most prevalent ESBL genes and it was beyond the scope of this study to determine other putative genes. In addition, this assay had a high sensitivity and negative predictive value (99.0% and 98.6%, respectively) compared with the CDT, which demonstrates a reliable performance for routine diagnostics, comparable to the currently used microarrays. Moreover, based on our findings, we believe that CDT probably leads to false-positive results, confirming the results of Garrec *et al.* in which the CDT gained a specificity ranging from 80% to 91% (depending on the antibiotics used) compared with PCR. Therefore, the specificity of the LM-PCR will probably be even greater than 92.2%.¹⁷

The LM-PCR analysis time of about 4.5 h (45 min DNA extraction, 2 h ligation, 1 h 40 min real-time PCR) is considerably shorter than that required for the CDT, as the latter requires an overnight incubation. The ability to detect transmissible antibiotic resistance, responsible for increased morbidity and mortality and prolonged hospital stays, one day earlier could positively influence the effectiveness of infection control.^{9,10,24} Rapid laboratory detection is necessary to take appropriate infection control measures, according to the Dutch guidelines for preventing nosocomial transmission of highly resistant micro-organisms. Moreover, rapid and correct detection of ESBLs is important to guide correct antimicrobial therapy.^{24,25}

Although the use of LM-PCR is more expensive than CDT (catalogue price €19.80 versus ~€1.20 per sample, respectively), it should be emphasized that the substantial number of false-positive ESBL results using the CDT will lead to unnecessary isolation of patients. It has been calculated that isolation of a patient means an additional €26.34 each day on a regular ward in a non-outbreak situation.²⁶

Looking at other recently developed molecular methods to detect the most prevalent ESBLs, LM-PCR has several advantages. For example, the turnaround time of the LM-PCR (~4.5 h) is

shorter than that of the microarray Check-ESBL assay (6.5–8 h, depending on the number of samples).^{8,13} Both Voets *et al.* and Dallenne *et al.* evaluated a conventional set of (multiplex) PCRs to detect the most important beta-lactamases without the use of an internal control.^{11,12} These methods, as well as the microarray system, have a large disadvantage for routine application because of a risk of contamination due to a post-amplification step (using amplification products for gel electrophoresis or microarray hybridization) that is needed for detection. Under routine laboratory conditions, these tests are applied on cultured strains; therefore, detection with a closed amplification system such as real-time PCR has major advantages.

In conclusion, the LM-PCR appears to be an accurate method to rapidly detect the most prevalent ESBLs, TEM, SHV and CTX-M. The assay can easily be introduced into any laboratory with real-time PCR equipment, allowing rapid detection of ESBLs. In fact, with only a few laboratory workflow adaptations, a new important reduction in turnaround time for molecular ESBL screening can be established. As a consequence, all ESBL results would be available within 1 day, making this assay an important tool for rapid and accurate ESBL detection.

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DISTRIBUTION OF EXTENDED-SPECTRUM BETA-LACTAMASE GENES USING A COMMERCIAL DNA MICRO-ARRAY SYSTEM

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Abstract

Extended-spectrum beta-lactamase (ESBL) genes are distributed worldwide and their epidemiology is complex. Using the Check-ESBL assay, the distribution of class A ESBL genes in clinical isolates of aerobic Gram-negative bacilli from three laboratories in the East of The Netherlands was determined. Four patient categories were distinguished: (i) patients admitted to an intensive care unit (ICU); (ii) non-ICU inpatients; (iii) outpatients admitted less than a year before collection of the isolate, (<1); (iv) outpatients admitted more than one-year prior to isolate collection or who had never been hospitalized (>1). From February 2009 until March 2010, out of 491 putative ESBL-positive isolates detected by the Vitek2 or Phoenix automated sensitivity testing systems, ESBL genes were detected in 247 (50.3%) by the Check-ESBL assay. Of these, 116 were from hospitalized patients (35 ICU, 81 non-ICU) and 131 were from outpatients (43 <1 , 88 >1). In all, 274 ESBL genes were identified in these 247 isolates: 153 CTX-M-1 group (predominantly in *E. coli* and *K. pneumoniae*, 70.4% and 51.6% respectively), 67 CTX-M-9 group (predominantly in *E. cloacae*, 57.9%), 32 SHV, 14 TEM and 8 CTX-M-2 group. ESBL-producing *E. cloacae* were significantly more common in hospitalized patients than in outpatients, 20.7% and 3.8% respectively ($P=0.001$). CTX-M-9 group ESBLs were significantly more prevalent in ICU patients ($P=0.003$), whereas SHV ESBLs were more common in hospitalized patients than in outpatients ($P<0.001$). There was no significant difference in distribution of ESBL genes between the two outpatient groups.

Introduction

Extended spectrum beta-lactamases (ESBLs) are plasmid-encoded enzymes, found frequently in *Enterobacteriaceae* and other Gram-negative organisms and which can hydrolyze beta-lactam drugs including third-generation cephalosporins. The most frequently detected and clinically important ESBLs belong to the TEM, SHV and CTX-M families.¹ TEM and SHV ESBLs derive by one or more point-mutations within non-ESBL TEM and SHV genes, whereas all CTX-M enzymes are ESBLs. CTX-M ESBLs can be divided into five groups by amino acid sequence similarities (groups 1, 2, 8, 9 and 25). Since the emergence of ESBLs in the early 1980s their prevalence has increased rapidly worldwide, both in the community and in hospitals.² Several studies have been performed to assess the frequency and distribution of ESBL genes, often using (real time) polymerase chain reaction (PCR), and regional differences in distribution have been demonstrated.³⁻⁸

The aim of this study was to describe the distribution of ESBL genes in our population, using a relatively new method of microarray analysis; the Check-ESBL assay (Check-Points Health BV, Wageningen, The Netherlands). During the 13-month study period, the Check-ESBL assay was used for genotypic detection and characterization of ESBL genes on all isolates which screened as ESBL positive by either Vitek 2 (BioMérieux, Marcy-l'Etoile, France) or the Phoenix system (BD Diagnostics, San Diego, CA, USA).

Materials and methods

Clinical isolates

The isolates studied were collected prospectively at three clinical microbiology laboratories in the East of The Netherlands which provide diagnostic services to five hospitals and a total population of 900,000 inhabitants: Department of Medical Microbiology and Medical Immunology, Rijnstate, Velp (A); Department of Medical Microbiology, Gelderse Vallei Hospital, Ede (B) and Department of Medical Microbiology, Hygiene and Infection Prevention, Slingeland Hospital, Doetinchem (C). Consecutive non-duplicate isolates from hospitalized patients, emergency departments, outpatient clinics, long term care facilities and general practice were included.

From February 2009 until March 2010 all phenotypically identified aerobic Gram-negative bacilli which screened positive for ESBL production according to the Clinical and Laboratory Standards Institute (CLSI) guidelines by either Vitek2 (BioMérieux, Marcy-l'Etoile, France), used by laboratories A and C, or the Phoenix system (BD Diagnostics, San Diego, CA, USA), used by laboratory B, were included. Isolates originated from both clinical specimens and surveillance specimens. Only the first of each ESBL-positive species from each patient was studied. From eight patients multiple species were isolated, of which each first ESBL-positive species isolate was included. Strains phenotypically suspected of ESBL production were further characterized using the Check-ESBL assay. Four patient categories were distinguished: (i) hospitalized patients

who were admitted to an intensive care unit (ICU), (ii) hospitalized patients admitted to hospital departments other than an ICU, (iii) outpatients who had been hospitalized within one year before collection of the isolate, and (iv) outpatients who had never been hospitalized or had been hospitalized more than one year before collection of the isolate.

Check-ESBL assay

DNA extraction was carried out using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). ESBL testing using the Check-ESBL assay was performed weekly in laboratory A. DNA extraction and the Check-ESBL assay were performed according to the manufacturers' instructions.

Briefly, the Check-ESBL assay is based on ligation-mediated amplification combined with detection of amplified product using a micro-array. Use of a well-developed micro-array allows three isolates to be tested at once. Images of the micro-array are generated using a reader (ArrayTube Reader, Clondiag Chip technologies, Jena, Germany) supported by software provided by the manufacturer. The assay indicates whether TEM or SHV-wildtype or TEM, SHV or CTX-M ESBL variants were present, and also differentiates CTX-M groups 1, 2, 9 and 8 in combination with 25.^{1,9,10} Analysis time is about 6-6.5 h.

Statistical analysis

Statistical significance for comparison of proportions was calculated by the chi-squared test using SPSS software version 14.0 ($P < 0.05$ was considered statistically significant).

Results

During the study period 491 putative ESBL-producing isolates were identified, 247 (50.3%) of which yielded a positive result in the Check-ESBL assay. Of these, 161 isolates (65.2%) were cultured from urine specimens, 28 (11.3%) from surveillance cultures (26 rectal swabs, one perineal swab and one faecal specimen), 20 (8.1%) from respiratory specimens, 13 (5.3%) from blood cultures and 25 (10.1%) from other clinical specimens. Among the 247 isolates were 179 *E. coli* (72.5%), 29 *Enterobacter cloacae* (11.7%), 26 *Klebsiella pneumoniae* (10.5%), five *Klebsiella oxytoca* (2.0%), four *Citrobacter freundii* (1.6%), one non-fermenter (0.4%), one *Enterobacter aerogenes* (0.4%), one *Proteus mirabilis* (0.4%) and one *Raoultella* spp. (0.4%). Table 1 shows the species identification of the Check-ESBL-positive isolates in total and in each of the three laboratories.

TABLE 1 Identification of Check-ESBL assay-positive isolates collected at the three clinical microbiology laboratories

Isolate	Hospital A (n=128)	Hospital B (n=73)	Hospital C (n=46)	Total (n=247)
<i>Escherichia coli</i>	68.0%	80.8%	71.7%	72.5%
<i>Enterobacter cloacae</i>	17.2%	1.4%	13.1%	11.7%
<i>Klebsiella pneumoniae</i>	8.6%	15.0%	8.7%	10.5%
<i>Klebsiella oxytoca</i>	3.1%	1.4%	-	2.0%
Other	3.1%	1.4%	6.5%	3.3%

ESBL, Extended-spectrum beta-lactamase

The 247 isolates were collected from 236 patients; 228 (96.6%) patients yielded one isolate for inclusion whereas eight (3.4%) patients yielded multiple species (six patients with two species, one with three, and one with four). Thirty-five (14.2%) isolates were collected from patients admitted to an ICU, 81 (32.8%) were collected from hospitalized patients admitted to departments other than the ICU, 43 (17.4%) were collected from outpatients who had been hospitalized within one year before collection of the isolate, and 88 (35.6%) were collected from outpatients who had either never been hospitalized or had been hospitalized more than one year earlier.

TABLE 2 Distribution of ESBL-producing species in the four patient categories

Isolate	Hospitalized patients		Out-patients	
	ICU (n=35)	Other than ICU (n=81)	Hospitalized <1 year before (n=43)	Never hospital- ized or >1 year before (n=88)
<i>Escherichia coli</i>	51.5%	62.9%	79.1%	86.4%
<i>Enterobacter cloacae</i>	20.0%	21.0%	2.3%	4.5%
<i>Klebsiella pneumoniae</i>	11.4%	9.9%	16.3%	8.0%
<i>Klebsiella oxytoca</i>	5.7%	3.7%	-	-
Other	11.4%	2.5%	2.3%	1.1%

ESBL, Extended-spectrum beta-lactamase; ICU, Intensive care unit

In all four patient categories *E. coli* was the most prevalent ESBL-producing Gram-negative bacillus (Table 2). The proportion of *E. cloacae* isolates was significantly higher in hospitalized patients than in outpatients, 20.7% (24/116) vs 3.8% (5/131) ($P=0.001$). Twenty-two (75.9%)

of the 29 *E. cloacae* isolates were isolated at laboratory A.

The 247 isolates yielded 274 distinct ESBL gene types, distinguished as follows: 153 (55.8%) CTX-M-1 group, 67 (24.5%) CTX-M-9 group, 32 (11.7%) SHV, 14 TEM (5.1%), and eight (2.9%) CTX-M-2 group. In 25 (10.1%) isolates more than one ESBL gene was identified: 22 isolates contained two ESBL genes and three isolates contained three ESBL genes.

In *E. coli* and *K. pneumoniae*, CTX-M-1 group ESBL genes were predominant, representing 70.4% and 51.6% of the genes identified in these species respectively. In *E. cloacae* CTX-M-9 group ESBL genes were predominant and represented 57.9% of all identified genes in this species. The distribution of ESBL genes in different species is shown in Table 3.

TABLE 3 Distribution of ESBL genes by species

Isolate	TEM	SHV	CTX-M1	CTX-M2	CTX-M9	CTX-M9 + SHV	CTX-M1 + M9	CTX-M1 + SHV	CTX-M1 + M9 + SHV	CTX-M1 + TEM
<i>E. coli</i> (n=179)	6.1%	1.7%	70.9%	4.5%	13.4%	1.1%	0.6%	0.6%	0.6%	0.6%
<i>E. cloacae</i> (n=29)		17.2%	6.9%		48.3%	20.7%	3.4%		3.4%	
<i>K. pneumoniae</i> (n=26)	3.8%	11.5%	53.8%		11.5%	11.5%	7.7%			
<i>K. oxytoca</i> (n=5)			20.0%			80.0%				
<i>C. freundii</i> (n=4)		25.0%			75.0%					
<i>E. aerogenes</i> (n=1)	100%									
Nonfermenter (n=1)						100%				
<i>P. mirabilis</i> (n=1)			100%							
<i>Raoultella</i> spp. (n=1)						100%				

Figure 1 shows the distribution of ESBL genes in isolates from the four patient categories. CTX-M-9 group was significantly more prevalent in isolates from patients admitted to an ICU compared with other patient categories ($P=0.003$). SHV ESBLs were significantly more frequent in isolates from hospitalized patients (ICU and non-ICU) than in isolates from outpatients ($P < 0.001$). The distribution of ESBL genes was not significantly different between the two outpatient categories.

Of the 35 isolates collected from patients admitted to an ICU, 20 were collected at hospital A, four at hospital B and 11 at hospital C. Of the 20 isolates from hospital A, CTX-M-9 group enzymes were present in nine, CTX-M-1 group in seven, SHV in two, TEM in one and CTX-M-2 group in one. All four isolates collected in hospital B were *E. coli* harboring CTX-M-1 group ESBL. The 11 isolates collected in hospital C consisted of eight with CTX-M-9 group ESBLs, two with CTX-M-1 group ESBLs and one with both.

FIGURE 1 Distribution of extended-spectrum beta-lactamase genes in the four patient categories. ICU, intensive care unit

Discussion

ESBLs are emerging worldwide in isolates derived both from the community and from hospitals. Their epidemiology has altered with time; until the end of the 1990s most were SHV and TEM types and isolates expressing these enzymes were associated with nosocomial outbreaks mainly in ICUs.¹¹ Now, *E. coli* expressing CTX-M beta-lactamases are the most widespread.¹¹ The epidemiology of ESBL genes is complex because there is both clonal spread of resistant strains and spread of specific plasmids and/or other mobile genetic elements. The distribution of ESBLs is far from uniform and there are considerable geographical differences in the prevalence of ESBL.¹²

The recently introduced Check-ESBL assay can accurately detect and identify the most

prevalent TEM, SHV and CTX-M ESBLs.^{1,9,10} The technique enables routine clinical microbiology laboratories to confirm the presence of ESBLs genotypically and to analyze the distribution and emergence of (specific) ESBL genes.

With the Check-ESBL assay, ESBL genes could be identified in 247 of the 491 (50.3%) isolates screened as ESBL positive by the Vitek2 or Phoenix systems. ESBL genes were identified in 46.4% (174/375) of isolates screened ESBL positive by Vitek2, and in 62.9% (73/116) of isolates screened ESBL positive by Phoenix. This might indicate that the Check-ESBL assay has poor performance but evaluations have previously shown good sensitivity and specificity (respectively 95% and 100%) when compared to PCR and sequencing.⁹ Other studies have demonstrated poor identification of ESBL using automated systems. For instance in a study by Sturm *et al.* only 43 of the 102 (42%) *E. coli*, *K. pneumoniae* and *K. oxytoca* isolates identified as ESBL producers using Phoenix were confirmed by PCR and sequence analysis.¹³ Both Thomson *et al.* and Wiegand *et al.* compared the detection of ESBLs with Vitek2 and Phoenix and reported poor sensitivities and specificities for both systems, Phoenix 52% and 58% respectively, Vitek2 78% and 85% respectively.^{14,15}

Although the aim of this study was not to discuss the sensitivity and specificity of the Check-ESBL assay as compared to phenotypic ESBL tests, all 491 isolates were tested routinely using a phenotypic ESBL test, the combination disc test (CDT). In 39 (7.9%) of the 491 isolates tested, a Check-ESBL-negative and CDT-positive result was obtained. These discrepant results might be explained by the presence of hyperproducing beta-lactamase genes such as K1-hyperproducers (often found in *K. oxytoca*) or by the occurrence of AmpC (often seen in *Enterobacter* spp. or *Serratia* spp., for example). On the other hand one cannot exclude the possibility of a false-negative Check-ESBL result in some of these 39 cases since the Check-ESBL assay detects TEM, SHV, CTX-M ESBL genes, but it does not detect the rarely found ESBL genes such as OXA, PER, VEB, GES. Because the scope of this study was to describe the distribution of ESBL genes in our population using the Check-ESBL assay and the assay already has been demonstrated to be an adequate method for detecting and characterizing ESBL genes, no other 'gold standard' was used in this study.⁹

The ESBL genes detected in this study were similar to those detected in other studies from Europe and the USA. Al Naiemi *et al.* reported in 2006 that CTX-M enzymes were predominant in Amsterdam, The Netherlands.¹² In the study by Sturm *et al.*, which described a population in close proximity to our own, CTX-M (43.5%) and TEM (39.1%) enzymes were predominant in *E. coli*.¹³ In the current study CTX-M enzymes were identified in 92.2% of the *E. coli* isolates whereas TEM enzymes were detected in only 6.7%. These observations suggest marked differences in the distribution of ESBLs even among geographically closely located populations, although Sturm *et al.* did not report whether their isolates were collected from hospitalized or non-hospitalized patients, so comparison is difficult.¹³

In general, as in our study, a predominance of CTX-M-1 group ESBLs is reported both in hospital and community-acquired isolates.¹⁶⁻¹⁹ However, in some studies non-CTX-M-1 ESBL types, for example CTX-M-9, CTX-M-2 or TEM or SHV ESBLs were more prevalent.³⁻⁸

Next to *E. coli* the most frequent species from hospitalized patients was *E. cloacae*, which was detected significantly more in hospitalized than non-hospitalized patients. *E. cloacae* is considered an emerging and increasingly important nosocomial pathogen, especially in ICUs.^{20–22} The most frequent ESBL in *E. cloacae* were CTX-M-9 group, SHV or a combination of the two (Table 3). This pattern has been reported elsewhere among *E. cloacae*: in hospitals in the UK, Germany and South Korea CTX-M-9 (also in combination with SHV) ESBLs were most prevalent in this species.^{22–24}

Comparing the ESBL types found in patients admitted to an ICU with the ESBL types found in patients admitted to other hospital departments, a distinct difference was found. Whereas CTX-M-9 group ESBLs were most prevalent in ICU isolates, CTX-M-1 group ESBLs were the most prominent in all other hospital isolates (Figure 1).

The outpatient group was arbitrarily distinguished according to whether or not patients had been hospitalized within the previous year, since comparison of these groups might indicate whether or not hospital-acquired ESBLs are able to reside in the community. No significant difference was found between these groups, but there was a significant difference in distribution between hospitalized patients and outpatients. These results might reflect loss of hospital-acquired ESBLs and/or replacement by community-acquired ESBLs within one year.

Since this study only involved three hospitals from the east of The Netherlands, extrapolation of the results to other countries would be inappropriate. However, the Check-ESBL assay makes it possible for any laboratory to detect the most prevalent ESBL types in its region. The assay has been found to be an important tool for monitoring the distribution of ESBL genes in our region.

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4

A RAPID AND HIGH-THROUGHPUT SCREENING APPROACH FOR METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* BASED ON THE COMBINATION OF TWO DIFFERENT REAL-TIME PCR ASSAYS

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen that has been responsible for major nosocomial epidemics worldwide. For infection control programs, rapid and adequate detection of MRSA is of great importance. We developed a rapid and high-throughput molecular screening approach that consists of an overnight selective broth enrichment, followed by *mecA*, *mecC*, and *S. aureus*-specific (SA442 gene) real-time PCR assays, with subsequent confirmation using a staphylococcal cassette chromosome *mec* element (SCC*mec*)-*orfX*-based real-time PCR assay (GeneOhm MRSA assay) and culture. Here, the results of the screening approach over a 2-year period are presented. During this period, a total of 13,387 samples were analyzed for the presence of MRSA, 2.6% of which were reported as MRSA positive. No MRSA isolates carrying the *mecC* gene were detected during this study. Based on the results of the real-time PCR assays only, 95.2% of the samples could be reported as negative within 24 h. Furthermore, the performance of these real-time PCR assays was evaluated using a set of 104 assorted MRSA isolates, which demonstrated high sensitivity for both the combination of *mecA* and *mecC* with SA442 and the BD GeneOhm MRSA assay (98.1% and 97.1%, respectively). This molecular screening approach proved to be an accurate method for obtaining reliable negative results within 24 h after arrival at the laboratory and contributes to improvement of infection control programs, especially in areas with a low MRSA prevalence.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen that has been detected throughout the world.^{1–3} MRSA has been responsible for major hospital epidemics, both regionally and globally.^{3,4} The introduction of the so-called “search and destroy” (S&D) policy in countries such as the Netherlands and Norway has proven to be of great importance in preventing the spread of MRSA, since the prevalence of MRSA in these countries is very low.^{5,6} Moreover, this strategy has been shown to save money and lives, which illustrates even more the impact of the S&D policy.⁷ Rapid diagnostic testing for MRSA is helpful for the S&D policy and will reduce the number of isolation days.

The Dutch guideline on the detection of MRSA recommends the use of a MRSA screening agar after overnight incubation with a nonselective broth, followed by molecular confirmation.⁸ With this method, it takes at least 72 h to obtain a (negative) result. Although culture remains the gold standard for detection of MRSA, several other approaches have been developed to detect the presence of MRSA. As early as 1991, the evaluation of a PCR targeting the *mecA* gene was published.⁹ In combination with a species-specific identification test for *S. aureus*, MRSA can be distinguished from methicillin-resistant coagulase-negative staphylococci (MRCNS), which also may contain the *mecA* gene. Such a species-specific PCR assay for *S. aureus* might be, for instance, a PCR targeting the *nuc* gene or the SA442 gene.^{10,11} However, the combination of a *mecA* PCR and a *S. aureus*-specific PCR may result in false-positive results when used in nonsterile samples that can contain both methicillin-susceptible *S. aureus* (MSSA) and MRCNS. Therefore, Huletsky *et al.* developed a real-time PCR assay that is based on the integration site of the staphylococcal cassette chromosome *mec* element (SCC*mec*) cassette, containing the *mecA* gene, and the *S. aureus*-specific *orfX* gene (SCC*mec*-*orfX*).¹² Rapid diagnostic assays, such as the Xpert MRSA assay (Cepheid, Sunnyvale, CA, USA) and the GeneOhm MRSA assay (BD Diagnostics, San Diego, CA, USA), are based on the principles of Huletsky *et al.* and are widely used for rapid detection of MRSA.^{13,14} Unfortunately, the SCC*mec*-*orfX*-based assays have proven not to be able to detect all MRSA isolates correctly. In the past, *mecA* dropouts have been reported, resulting in false-positive MRSA results.^{12,15} Moreover, because of the high diversity in SCC*mec*-*orfX* sequences, correct detection remains a challenge.^{16,17} The recent discovery of the *mecC* gene, which cannot be detected by both the *mecA* assays and the SCC*mec*-*orfX*-based assays, makes the detection of MRSA even more difficult.¹⁸ Since both the combination of *mecA*-*mecC* together with an *S. aureus*-specific real-time PCR assay and the SCC*mec*-*orfX*-based approach have their shortcomings when used as a single assay to detect MRSA in clinical samples, we decided to combine the two assays to acquire a rapid and high-throughput molecular MRSA screening tool.

Here, we present the results of our molecular screening approach for the detection of MRSA, which consists of an overnight broth enrichment, followed by *mecA*, *mecC*, and a *S. aureus*-specific (SA442) real-time PCR assay, with subsequent confirmation using an SCC*mec*-*orfX*-based real-time PCR assay and culture. The present report describes the results of this approach over a period of 2 years (2012 and 2013).

Materials and methods

Samples and DNA extraction

All samples for MRSA screening from 10 January 2012 to 31 December 2013 were inoculated into a phenyl mannitol broth (PMB) containing ceftizoxime (5 µg/ml) and aztreonam (75 µg/ml) for overnight incubation ($35 \pm 2^\circ\text{C}$).¹⁹ Nose and throat swabs from each patient were pooled, while perineal swabs and all other possible samples (such as wound swabs, urine, or sputa) were processed separately. After at least 16 h of incubation, the broths were mixed thoroughly, and 100 µl of broth was used for DNA extraction. In short, 100 µl broth was mixed well with 100 µl (1 U/µl) achromopeptidase (Sigma-Aldrich Co., St. Louis, MO, USA) and 20 µl internal inhibition control (IC) (phocine herpesvirus, PhHV-1).²⁰ After incubation for 15 min at 37°C , followed by 5 min at 95°C and centrifugation (1 min; 14,000 rpm), the supernatant was used for detection of MRSA.

Real-time PCR assays

Two internally controlled real-time PCR assays were performed: the first assay detects the *S. aureus*-specific SA442 gene, while the second targets the *mecA* gene and the *mecC* gene (together forming the SMM panel). The sequences of all the primers and probes are shown in table 1. Both assays were simultaneously performed in about 1 h 40 min on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA) in a 30-µl volume containing 10 µl supernatant, primers, and probes as shown in table 1 and 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The real-time PCR conditions were 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15s and 60°C for 60s.

A sample was suspected to be MRSA positive when the SA442 PCR resulted in a threshold cycle (Ct) value of 39 or lower in combination with a positive *mecA* or *mecC* PCR (Ct value, <45). Samples with a Ct value of >39 for the SA442 PCR were interpreted as negative, since an in-house validation showed that no *S. aureus* isolate was cultured when a Ct value of >38 was found (data not shown). Samples were interpreted as inhibited when the Ct value of the IC was >2 Ct values higher than the IC of a negative isolation control. For these samples, DNA extraction and the SMM panel were repeated with a 10-fold dilution of the broth. Interpretation of these diluted samples was performed as described above. Samples showing inhibition after dilution were cultured.

TABLE 1 Primer- and probe sequences of the SMM-panel

Oligo	Sequence (5'-> 3')	Final concentration	Origin
SA442-forward	CAATCTTTGTCGGTACACGATATCT	333nM	Modification of Martineau et al. ¹¹
SA442-reverse	CAACGTAATGAGATTCAGTAGATAATACAAC	333nM	
SA442-probe 1	FAM – CACGACTAAATAAACGCTCATTCGCGATTTT – BHQ1	150nM	Developed by E.J. van Hannen
SA442-probe 2	FAM – CACGACTAAATAGACGCTCATTCGCAATTTT – BHQ1	150nM	
IC-forward	GGG CGA ATC ACA GAT TGA ATC	333nM	Van Doornum et al. ²⁰
IC-reverse	GCG GTT CCA AAC GTA CCA A	333nM	
IC-probe	CY5 - TTT TTA TGT GTC CGC CAC CAT CTG GAT C – BHQ2	150nM	
MecA-forward	GATTATGGCTCAGGTACTGCTATCC	333nM	Developed by E.J. van Hannen
MecA-reverse	TTCGTTACTCATGCCATACATAAATG	333nM	
MecA-probe	VIC - CCCTCAAACAGGTGAATTATTAGCACCTTGAAGCA – BHQ1	250nM	
MecC-forward	GCA AGC AAT AGA ATC ATC AGA CAA C	333nM	Developed by E.J. van Hannen
MecC-reverse	TCT TGC ATA CCT TGC TCA AAT TTT	333nM	
MecC-probe	NED - CCG CAT TGC ATT AGC ATT AGG AGC CA – BHQ2	100nM	
IC-forward	GGG CGA ATC ACA GAT TGA ATC	333nM	Van Doornum et al. ²⁰
IC-reverse	GCG GTT CCA AAC GTA CCA A	333nM	
IC-probe	CY5 - TTT TTA TGT GTC CGC CAC CAT CTG GAT C – BHQ2	150nM	

FAM, 6-carboxyfluorescein; BHQ, black hole quencher; NED, 2'-chloro-5'-fluoro-7',8'-fused phenyl-1,4-dichloro-6-carboxyfluorescein; VIC, 6-carboxyrhodamine.

As illustrated in figure 1, in case of a suspected MRSA-positive PCR result in the SMM panel, a second real-time PCR assay (GeneOhm MRSA; BD Diagnostics, San Diego, CA, USA) was performed on the vast majority of samples. Only when a *mecC* signal in combination with the SA442 signal was detected or when the suspected MRSA-positive sample (a positive result in the SMM panel) was sent by a general practitioner, that is, when rapid results of MRSA detection were less urgent and costs could be reduced, was the broth subcultured directly without the second PCR step, as described below. The GeneOhm MRSA assay was performed on the Smartcycler system (BD Diagnostics, San Diego, CA, USA) by adding 3 μ l supernatant to 25 μ l of master mix provided with the kit, as instructed by the manufacturer. After a run of about 1 h, interpretation was performed by the software, and the results were reported as positive, negative, or unresolved (inhibition). Each sample that was either positive or unresolved was presented for culture. Moreover, samples negative in the GeneOhm MRSA assay that were suspected to contain livestock-associated MRSA (LA-MRSA), as stated by the applicant, were cultured.

Culture and spa typing

The broth was subcultured using a 1- μ l loop onto a Brilliance MRSA2 (Oxoid Ltd., Basingstoke, United Kingdom) and 5% sheep blood agar (Oxoid) and analyzed after overnight incubation ($35 \pm 2^\circ\text{C}$). Suspected colonies were captured and identified as *S. aureus* using matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany). Methicillin resistance was confirmed with a ceftioxin disc (30 μ g) and Vitek2 (BioMérieux, Marcy l'Etoile, France). Each new MRSA isolate was sent to the Dutch National Reference Center (RIVM, Bilthoven, the Netherlands) to determine the *spa* type of the isolate.

Evaluation of the real-time PCR assays

In order to assess the performance of both the SMM panel and the GeneOhm MRSA assay, a total of 101 assorted MRSA isolates of unknown origin and 3 reference strains were tested (table 2). All isolates were suspended in 0.45% saline (NaCl) up to a density of approximately 0.7 McFarland standard. After a short centrifugation step, the supernatant was tested using the different real-time PCR assays.

FIGURE 1 Schematic overview of the MRSA screening approach.

GP, samples obtained from patients of general practitioners

TABLE 2 Overview of results obtained evaluating the real-time PCR assays using test strains

<i>spa</i> type	N	SA442	<i>mecA</i>	<i>mecC</i>	BD GeneOhm
t002	3	3	3	0	3
t003	2	2	2	0	2
t008	14	14	14	0	14
t011 ^a	26	26	26	0	26
t015	1	1	1	0	1
t019	8	8	8	0	8
t022	1	1	1	0	1
t034 ^a	7	7	7	0	7
t044	1	1	1	0	1
t064	8	8	8	0	8
t105	1	1	1	0	1
t108 ^a	6	6	6	0	6
t189	2	0	2	0	1
t202	1	1	1	0	1
t223	2	2	2	0	2
t267	1	1	1	0	1
t296	1	1	1	0	1
t311	1	1	1	0	1
t316	1	1	1	0	1
t318	1	1	1	0	1
t359	2	2	2	0	2
t437	1	1	1	0	1
t548	1	1	1	0	1
t571 ^a	1	1	1	0	1
t688	1	1	1	0	1
t786	1	1	1	0	1
t791	1	1	1	0	1
t899 ^a	1	1	1	0	0
t1456 ^a	1	1	1	0	1
t1814	1	1	1	0	1
t3932	1	1	1	0	1
t9633	1	1	1	0	1
ATCC 43300 (t007)	1	1	1	0	1
NCTC 10442 (t008)	1	1	1	0	1
<i>mecC</i> (t7603) ^a	1	1	0	1	0
Total	104	102 (98.1%)	103 (100%)	1 (100%)	101 (97.1%)

^a Livestock-associated MRSA (ST398)

Results

Real-time PCR assays

During a 2-year period, a total of 13.387 samples were presented for MRSA screening and tested using the SMM panel (table 3). Of these samples, 2.112 (15.8%) were defined as suspected MRSA positive based on the presence of the SA442 gene (Ct value ≤ 39) in combination with the presence of the *mecA* gene. In none of the samples was the *mecC* gene detected. Of all samples tested with the SMM panel, only 6 (0.04%) were inhibited after dilution.

Of the 2.112 suspected MRSA-positive samples, 1.858 (88%) were subsequently tested with the GeneOhm MRSA assay, of which 365 samples were positive and 17 had an unresolved result. The remaining 254 samples (12.0%) were sent by a general practitioner and were therefore subcultured directly. Overall, based on the results of both real-time PCR assays, a total of 12.745 (95.2%) samples could be reported as negative within 24 h.

TABLE 3 Results of the screening approach

	SMM-panel			GeneOhm MRSA assay						Culture		
	Tested ^a	Positive	Unresolved	Tested ^a	Positive	Unresolved	Tested ^a	Positive	Unresolved	Tested ^a	GeneOhm MRSA assay positive	GP
2012	4,117 (2,040)	557	3	430 (306)	76	5	76 (39)	50		127 (87)		31
2013	9,270 (5,708)	1,555	3	1,428 (1,166)	289	12	288 (184) ^b	239		127 (95)		30
Total	13,387 (7,748)	2,112	6	1,858 (1,472)	365	17	364 (223)	289		254 (182)		61

GP, general practitioner.

^a In parentheses, the number of pooled samples tested.

^b One GeneOhm MRSA assay-positive sample could not be cultured.

MRSA culture and *spa* typing

Of the 13.387 samples received for MRSA screening by real-time PCR, 642 samples (4.8%) were eventually cultured. This was either due to a positive result in both the SMM panel and the GeneOhm MRSA assay ($n = 365$), due to a positive result in the SMM panel in combination with the fact that they were sent in by a general practitioner ($n = 254$), or due to inhibition in the real-time PCR assays ($n = 23$). Seven additional samples had to be cultured because of suspicion of LA-MRSA, for which the SMM panel was positive and the GeneOhm MRSA assay remained negative.

Out of the 365 samples that were presented for culture because of the positive interpretation of the GeneOhm MRSA assay, one sample was not available for culture. Of the remaining 364 samples, 289 (79.4%), 121 of which eventually proved to be LA-MRSA, from 213 different patients were confirmed MRSA positive by culture.

Of the suspected MRSA-positive samples based on the SMM panel, 254 were sent by a general practitioner and were presented for culture directly. Sixty-one (24.0%) of these samples from 45 different patients were MRSA positive in culture.

Among the 23 samples that had to be cultured because of inhibition in the real-time PCR assays, 20 samples were available for culture, and all were determined to be MRSA negative.

Six out of the seven samples that were negative in the GeneOhm MRSA assay were cultured because of suspicion of LA-MRSA. In none of the cultured samples was MRSA found. Due to an error, the remaining sample could not be cultured.

In total, 350/13.387 samples (2.6%) were reported MRSA positive, 150 of which (42.9%) proved to be LA-MRSA, since the isolates belonged to sequence type 398 (ST398). The 350 MRSA-positive samples were obtained from 256 individuals.

Each new MRSA isolate was sent to the RIVM for *spa* typing. As shown in table 4, *spa* typing of 258 MRSA isolates showed 45 different types, with t011 the most prevalent, followed by t064. Two individuals had two different MRSA isolates, with *spa* types t011 and t899 found in the nose/throat samples and *spa* types t4119 and t4571 in the perineal samples.

Evaluation of the real-time PCR assays

In general, real-time PCR assays may result in false-negative results due to primer-probe mismatches. This is especially the case for SCC*mec-orfX*-based assays, where large variations in the primer-probe region are described. In order to assess the correct performance of the real-time PCR assays used in this method, a total of 101 assorted MRSA isolates of unknown origin detected at three different laboratories for medical microbiology, along with 3 reference isolates, were subjected to these PCR assays. All the MRSA isolates were correctly detected by both the *mecA* and the *mecC* real-time PCR assays. The real-time PCR targeting the *S. aureus*-specific gene SA442 was able to detect 102 (sensitivity, 98.1%) of the MRSA isolates. The two SA442-negative isolates both belonged to *spa* type t189. Moreover, one of these samples was not detected by the GeneOhm MRSA assay. Furthermore, the GeneOhm MRSA assay was not able to detect an isolate with *spa* type t899, as well as the isolate with the *mecC* gene (table 2).

TABLE 4 *spa* types detected during the study

<i>spa</i> type ^a	2012	2013	Total
t011 ^b	22	36	58
t064	2	33	35
t002	2	23	25
t008	5	19	24
t034 ^b	3	14	17
t899 ^b	5	7	12
t1081		12	12
t108 ^b	1	9	10
t032		9	9
t223		4	4
t1457 ^b		3	3
t179	2	1	3
t359		3	3
t105	2		2
t003	1	1	2
t044	1	1	2
t067		2	2
t1474		2	2
t2922 ^b	1	1	2
t437	2	1	3
t447		2	2
t571 ^b		2	2
t791	2		2
t019, t084, t211, t311, t324 t441, t786, t965 t1430, t1451 ^b , t1930, t2011 ^b , t2330 ^b , t4119 ^b , t4132 ^b		All 1	15
t121, t127, t148, t304, t321, t443, t4571 ^b	All 1		7
Not determined	1		1
Total	59	200	259

^a All single *spa* types found per patient^b Livestock-associated MRSA (ST398)

Discussion

In this study, we evaluated our molecular screening approach for the detection of MRSA over a 2-year period. During this period, 13,387 samples were inoculated into a selective broth and tested in the SMM panel after an overnight incubation. Of these, 2,112 (15.8%) were defined as suspected MRSA positive, since the SA442 PCR showed a Ct value of 39 or lower in combination with a positive *mecA* PCR. In the vast majority of cases ($n = 1,858$), the GeneOhm MRSA assay was used as a confirmation for the samples that were suspected MRSA positive in the SMM panel. Culture was performed from a total of 618 suspected MRSA-positive samples. Of these samples, 364 had a positive result using the GeneOhm MRSA assay, while the remaining 254 were subcultured directly following the SMM panel, since the samples originated from a general practitioner. Eventually, 350 samples (2.6%) were MRSA positive.

Using this molecular screening approach, 95.2% of the samples could be reported negative based on the real-time PCR assays. Both assays had shown good sensitivities and negative predictive values in earlier studies, making this screening approach very useful for detection of MRSA-negative samples.^{15,21}

As this approach is performed on a daily basis, results can be available within 24 h. Moreover, since the SMM panel has a capacity of 44 samples per real-time PCR run of 1 h 40 min, 176 samples (a total of 4 runs) can be analyzed per real-time PCR system within one working day. This high throughput makes the approach an extremely valuable tool during outbreak situations to monitor and adjust the infection control interventions. By skipping the SMM panel and performing the GeneOhm MRSA assay directly on the broth, the time to result would decrease from 2 h 40 min (combining the SMM panel and the GeneOhm MRSA assay) to 1 h. However, since the Smartcycler has a capacity of only 14 samples per real-time PCR run, only 98 samples (a total of 7 runs) can be analyzed per Smartcycler system within one working day. Moreover, the price per sample is approximately 8 times higher when the GeneOhm MRSA assay is used instead of the in-house SMM panel. Therefore, the strength of this approach is the combination of both real-time PCR assays, in which the SMM panel is associated with a high throughput and low cost. The surplus value of the GeneOhm MRSA assay was demonstrated because MRSA was cultured in 79.4% of all samples that tested positive by the GeneOhm MRSA assay, while the percentage was much lower when samples were cultured directly following the SMM panel (24%). Nevertheless, it should be stressed that positive results of both real-time PCR assays should always be confirmed using culture, since false-positive interpretations can be observed in the SMM panel and the GeneOhm MRSA assay because of a mixture of MRCNS and MSSA or a SCC*mec*-cassette with a *mecA* dropout, respectively.^{21–23}

For nonhospitalized patients, however, there is usually less need for rapid MRSA results than for hospitalized patients. To reduce costs, the GeneOhm MRSA assay was not applied for samples supplied by general practitioners; if the SA442 assay showed Ct values of 39 or lower in combination with a positive *mecA* or *mecC* PCR, broth samples were subcultured on Brilliance MRSA2 and blood agar plates immediately, as shown in figure 1.

Introduction of a dCt-based strategy has been shown to improve the sensitivity and specificity of MRSA detection using real-time PCR assays targeting an *S. aureus* species-specific gene and the *mecA* gene.^{24,25} Bode *et al.* applied this strategy in their method and focused on the difference in Ct values between SA442 and *mecA* (dCt, the Ct value of SA442 minus the Ct value of *mecA*).²¹ By introducing this dCt, they showed 97.5% sensitivity (39/40) and 96.6% specificity (1.701/1.760) when a dCt between –2 and +8 was assessed. Although the dCt can, for instance, be influenced by the use of different broths (with different [concentrations of] antibiotics), we did compare our data with the study of Bode *et al.*, since the same selective broth and target genes were used. In our study, the dCt varied from –10.13 to 14.9, with a median of –0.51. In our hands, the sensitivity would be only 86.3% (302/350) when the dCt between –2 and +8 was used for the results. Despite some minor differences between the two real-time PCR assays, we discourage the use of such a dCt for the interpretation of the SMM panel, since this may lead to false-negative results.

In general, false-negative results may occur using real-time PCR assays because of primer-probe mismatches. In order to assess the performance of the real-time PCR assays used in this method, a panel of 104 assorted MRSA isolates, consisting of 101 MRSA isolates of unknown origin and 3 reference strains, were subjected to the real-time PCR assays. This panel of isolates included the 5 most prevalent detected *spa* types in the Netherlands during 2008 and 2009 (t011 and t108 [both LA-MRSA] and t008, t002, and t064 [not LA-MRSA]).²⁶ While the sensitivity of the *mecA-mecC* PCR was 100%, the SA442 assay tested negative for 2 of the isolates (sensitivity, 98.1%). This is comparable to the findings of two studies that showed 99.7% and 99.9% sensitivity in panels of 2.600 and 4.500 *S. aureus* isolates, respectively.^{27,28} Although the sensitivity is very high, these results still might lead to undetected MRSA. The introduction of a second target gene for the detection of *S. aureus*, such as the *nuc* gene, might further improve the sensitivity. Although *nuc*-deficient isolates have sporadically been reported, combining the two genes for detection of *S. aureus* will be important in order to approach correct detection of all possible *S. aureus* strains.²⁹

The GeneOhm MRSA assay detected 97.1% of the isolates correctly. Among the 3 isolates that were determined to be negative, one isolate contained the *mecC* gene, known to be negative in this assay. Since in our approach the GeneOhm MRSA assay was abolished for *mecC*-positive isolates and broths were subcultured directly, no *mecC*-containing MRSA isolates among our clinical specimens would have been missed.¹⁸ Apart from the *mecC* isolate, false-negative results can be produced by this assay, especially in LA-MRSA, because of the variability in the *SCCmec* and/or *orfX* sequences.^{30,31} Therefore, samples in which LA-MRSA is suspected (as stated by the applicant) were subcultured even when a negative result was found using the GeneOhm MRSA assay.

Only 23 samples were inhibited in either the SMM panel or the GeneOhm MRSA assay. Three samples were lost; all other samples showing inhibition were further processed by culture and produced a negative result.

None of the samples contained the *mecC* gene, indicating a very low prevalence of MRSA containing *mecC* in the Netherlands, just as in, for instance, the United Kingdom, Germany, and Switzerland.^{32–35} In contrast to these findings, the prevalence of MRSA containing the *mecC* gene seems to be increasing in Denmark, indicating the need to screen for the *mecC* gene.³⁶

Due to several outbreaks in hospitals and nursing homes in 2013, an enormous increase in samples was observed, with 4.117 and 9.270 samples in 2012 and 2013, respectively. Hence, a high number of MRSA isolates with *spa* types t002, t008, t034, t064, and t1081 were found compared to 2012 (table 4). Overall, the most prevalent *spa* type was t011, followed by t064, t002, and t008. This is in accordance with the epidemiological data for 2008 and 2009 published by Lekkerkerk *et al.* and with the data shown in a report on the surveillance of MRSA in the Netherlands in 2011 available on the website of the RIVM (Bilthoven, the Netherlands) (https://mrsa.rivm.nl/jaarverslagen_mrsa).²⁶

The screening approach used differs from the Dutch guidelines on the laboratory detection of MRSA, which recommend the use of (relatively) nonselective broth enrichment in combination with subculture on a MRSA screening agar and nonselective blood agar plate for 48 h, yielding negative results no sooner than 72 h.⁸ Our approach consisted of an overnight incubation with PMB. To our knowledge, no head-to-head comparison between PMB and a nonselective broth has been reported, but Böcher *et al.* did show improvement of screening for MRSA when using a semiselective broth over a nonselective broth.³⁷ Although this result cannot be directly extrapolated to the performance of the selective broth used in our study (PMB) in comparison with a nonselective broth, it suggests a comparable improvement in detecting MRSA. Following broth enrichment, culture using a MRSA screening agar instead of molecular detection methods is recommended by the Dutch guidelines, since the authors found that evidence was too limited to recommend the use of broth enrichment in combination with molecular methods.⁸ Recently however, multiple studies have compared the use of broth enrichment in combination with real-time PCR assays to the combination of broth enrichment followed by culture methods. These studies have shown high sensitivities when using broth enrichment and subsequent real-time PCR, showing the reliability of the combined methods.^{21,24,25} Based on the published data in combination with the data here, we suggest that recommendations on the use of broth enrichment in combination with real-time PCR assays be included in the Dutch guidelines on the laboratory detection of MRSA.

In conclusion, this molecular screening approach for MRSA based on the combination of two different real-time PCR assays has proven to be highly sensitive and specific. Because of the great sensitivity and negative predictive values of the real-time PCR assays, reliable negative results can be obtained within 24 h after the samples arrive at the laboratory. Especially during outbreaks of MRSA, this high-throughput screening approach yielding rapid negative results can be a major improvement in infection control programs.

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5

NEW SCREENING METHOD TO DETECT CARRIAGE OF CARBAPENEMASE-PRODUCING *ENTEROBACTERIACEAE* IN PATIENTS WITHIN 24 HOURS

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Abstract

The prevalence of carbapenemase producing *Enterobacteriaceae* (CPE) is increasing worldwide at an alarming rate. Invasive infections with these strains are associated with high mortality rates. Rapid identification of colonized patients (carriers) is essential to prevent the spread of CPE in the hospital. Unfortunately, at the moment there are no conclusive guidelines how and who to screen. Here, we describe the clinical application of a new screening strategy to detect patients colonized with CPE. In March 2012 we routinely introduced this method to screen high risk patients for carriage of CPE. A high risk patient was defined as any patient who had been hospitalized abroad in the preceding two months. From these patients a rectal and throat swab were collected and incubated overnight in both an ertapenem and a ceftazidime broth. After incubation, DNA was isolated and tested for the most prevalent CPE genes (*bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{OXA-48}) by a ligation-mediated real time PCR. Between March 2012 and April 2013 454 patients were screened. In 6 patients CPE were detected by PCR, all of which were confirmed by subsequent culture (carriage rate 1.3%). We found 2 KPC and 6 OXA-48 isolates in patients who had been hospitalized in Morocco (2), Italy, Greece, Egypt and Jordan. In one patient 3 different OXA-48 isolates were detected. This accurate and rapid screening method proved to be a major improvement in the implementation of a surveillance program for CPE in our hospital.

Introduction

Carbapenemase producing *Enterobacteriaceae* (CPE) have emerged rapidly and extensively worldwide. This is an important source of concern for both the single patient as well as public health since only few antimicrobials are currently available to treat infections with CPE. Moreover, invasive infections with carbapenem resistant strains are associated with high mortality rates up to 40%-50%, emphasizing the need for fast and accurate detection.¹⁻⁴

In *Enterobacteriaceae*, carbapenem resistance is mainly caused by the production of carbapenemases; carbapenem-hydrolyzing beta-lactamases which lead to resistance to almost all beta-lactams. These carbapenemase enzymes belong to three molecular classes of beta-lactamases, Ambler class A (e.g. *bla*_{KPC}), class B (e.g. *bla*_{VIM}, *bla*_{NDM}, *bla*_{IMP}) and class D (e.g. *bla*_{OXA-48}).⁵

Since there has been documentation of high CPE prevalence in widespread geographical regions⁶, screening for asymptomatic carriers should at least be performed in all patients who have been hospitalized in a foreign hospital, as described in the national guideline 'highly-resistant micro-organisms' by the Working Party of Infection Prevention.⁷ The strategy as recommended in the Dutch guideline to screen for the presence of CPE is a two-step procedure. The strategy consists of a phenotypic screening step, based on the detection of reduced susceptibility to carbapenems, followed by a confirmation step. Here, preferably a genotypic confirmation step is performed able to detect the presence of carbapenemase genes. When this is not available, phenotypic confirmation can be performed using for example the Modified Hodge test.

Screening for gastrointestinal colonization of CPE has shown to be helpful in reducing the incidence and secondary transmission in the hospital.^{8,9} Unfortunately, there is no strong evidence on the best screening strategy to do so.¹⁰ This article presents the results of a new screening method to detect carriage of CPE in patients within 24 h during a 14-month period.

Materials and methods

Screening method

A rectal swab and a throat swab were collected and transported in Σ -transwab medium to the laboratory according to the manufacturers guidelines (MW&E, Corsham, UK). A volume of 100 μ l of the Σ -medium from the rectal swab and 100 μ l of the Σ -medium from the throat swab were incubated together in two different broths each (0.25 μ g/ml ertapenem with 50 μ g/ml vancomycin and 2 μ g/ml ceftazidime with 50 μ g/ml vancomycin). After overnight incubation (35 $\pm$ 2°C), 250 μ l of each of the two broths were merged and mixed well. Following a wash step and subsequent centrifugation (1min, 14000rpm), the pellet was suspended in 100 μ l of the enzyme achromopeptidase and 20 μ l internal inhibition control (IC). After incubation for 15 minutes at 37°C followed by 5 minutes at 95°C and centrifugation (1min, 14000rpm), the supernatant was used for detection of carbapenemase genes. To each run, a negative isolation

control was added to control possible inhibition in each sample.

Detection was performed using the Check-MDR Carba assay (Check-points, Wageningen, The Netherlands) that is able to detect the most prevalent carbapenemase genes in gram-negative bacteria (*bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{OXA-48}).^{11,12} The procedure was executed according to the manufacturers guidelines and consisted of two steps. In short, a ligation step (non-cyclic: 3min 95°C, 2hr 65°C, 2min 98°C) was performed using 5µl solution A, 2.5µl solution P and 10µl DNA. After the ligation step, real-time PCR was performed in a 30µl volume containing 2X TaqMan Universal Mastermix (Applied Biosystems, CA, USA), solution R (Check-Points, Wageningen, the Netherlands) and 5µl ligation product using the TaqMan 7500 system (Applied Biosystems, CA, USA). To exclude false positive results due to background signals (Ct values >37), a cut-off value was introduced using the well-titrated internal control. The Check-MDR Carba was considered positive when the carbapenemase signal (FAM-labeled probe) was stronger than the IC-signal (Ct value carbapenemase < Ct value IC) using a threshold of 0.05 and an automatic baseline (correcting the background fluorescence detected automatically). A sample was considered inhibited when the Ct value of the IC of the corresponding sample was more than 2 Ct values higher than the IC of the negative isolation control.

Culture

In case of a positive result with the Check-MDR Carba assay, both broths were subcultured on Columbia bloodagar and MacConkey agar (both Oxoid, Basingstoke, UK). The species were determined with Matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonik GmbH, Bremen, Germany) and antibiotic susceptibility was measured by Vitek2 (BioMérieux, Marcy l’Etoile, France) and confirmed by E-test. Strains were sent to the Dutch National Institute for Public Health and the Environment (RIVM) for identification of the specific carbapenemase.

Clinical application

After an in house validation (Rijnstate, Velp) the method was implemented for routine screening on CPE high-risk patients.¹³ We defined a high risk patient as any patient who had been hospitalized in a foreign hospital in the preceding two months. From these patients a throat and a rectal swab were collected. The detection of CPE was subsequently performed according to our screening method as described above.

TABLE 1 Continent of hospitalization

Continent	Number of screened patients	Number of positive patients
Africa	25	3
America	12	0
Asia	52	1
Europe	295	2
Northern	10	0
Eastern	15	0
Southern	123	2
Western	147	0
Total	384	6

Results

A total of 465 samples from 454 different patients who had been hospitalized abroad were screened between 1 March 2012 and 30 April 2013. Results for the detection of CPE using the Check-MDR Carba assay were all available within 24 hours. For 384 (85%) of the patients, the country of hospitalization was known. Most patients had been hospitalized in western and southern Europe (table 1).

In six of the 454 patients CPE were detected (carriage rate 1.3%). Five patients were colonized with one CPE and one patient with three different isolates harboring a carbapenemase gene. These patients were screened because of hospitalization in southern Europe (Italy, Greece), Africa (Morocco (two), Egypt) or Asia (Jordan). Patient data are recorded in table 2. Detected CPE were identified as five *Klebsiella pneumoniae* (three OXA-48, two KPC), two *Escherichia coli* (both OXA-48) and one *Enterobacter cloacae* (OXA-48). All but one isolate grew in both (ceftazidime and ertapenem) broths. The isolate detected only in the ertapenem broth was identified as an OXA-48 producing *E. coli* and was extended-spectrum beta-lactamase (ESBL) negative.

TABLE 2 Epidemiological data for detected carbapenemase-producing *Enterobacteriaceae* isolates

Patient's sex, age, country	Date	Isolate	Carbapenemase	MIC meropenem (µg/ml)	Patient information
Male, 60 years, the Netherlands	09-08-2012	<i>Klebsiella pneumoniae</i>	KPC	>32	Hospitalization for 2 weeks including 1 week ICU in Italy after an out of hospital reanimation.
Female, 61 years, Greece	05-11-2012	<i>Klebsiella pneumoniae</i>	KPC	>32	Hospitalization for 3,5 months including ICU in Greece because of respiratory failure due to an exacerbation of COPD. Before hospitalization already living in Greece for several years.
Male, 85 years, Morocco	27-12-2012	<i>Klebsiella pneumoniae</i>	OXA-48	12	Hospitalization including ICU in Morocco because of gastrointestinal bleeding. Before hospitalization already living in Morocco for several years.
Female, 79 years, Morocco	14-01-2013	<i>Enterobacter cloacae</i>	OXA-48	6	Hospitalization for 2-3 months in Morocco because of a CVA and a myocardial infarction. Before hospitalization already living in Morocco for several years.
Male, 58 years, the Netherlands	21-01-2013	<i>Klebsiella pneumoniae</i>	OXA-48	16	Hospitalization for 2,5 weeks in Jordan because of pneumonia.
Male, 53 years, the Netherlands	20-03-2013	<i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> (2x)	OXA-48	1.5, 2, 0.75, respectively	Hospitalization including surgery in Egypt for an acute herniated disc.

MIC, minimum inhibitory concentration; ICU, intensive care unit; COPD, chronic obstructive pulmonary disease; CVA, cerebrovascular accident.

Discussion

This article described the routine application of a new screening method to detect carriage of CPE in patients who had been hospitalized abroad. In this screening method, two different broths were used to incubate the swabs. The use of these broths was necessary to increase the sensitivity of the method, since especially the rectal swab harbors a high number of miscellaneous bacteria (data not shown). For the carbapenem containing broth, ertapenem was used. Ertapenem was selected since this antibiotic is more stable in storage than meropenem or imipenem and the minimum inhibitory concentration (MIC) values of ertapenem are usually higher than the MIC values of other carbapenems. Moreover, the use of ertapenem has earlier been reported to be highly sensitive for the detection of KPCs by susceptibility testing.^{14,15} The ceftazidime broth was added for the detection of VIM isolates because one of the VIM isolates used in the validation process (MIC for meropenem 4 µg/ml) did not grow sufficiently in the ertapenem broth (data not shown). Since VIM producers are always resistant to cephalosporins¹⁶, the ceftazidime broth was added to our method for the correct detection of the VIM isolates.

During the study period eight different CPE isolates were detected. Of these eight isolates, all except one could be cultured in both the ertapenem and ceftazidime broth. The exception was an *E. coli* harboring OXA-48 that could not be cultured in the ceftazidime broth. Since this particular isolate was tested ESBL negative, and Amber class D carbapenemases (including OXA-48) are known to spare the inactivation of extended-spectrum cephalosporins¹⁶, it was inhibited in the ceftazidime broth. Consequently, the combination of both broths is required to be able to detect all of the most important carbapenemases.

The Check-MDR Carba assay was selected since it was the only commercially available assay able to detect all most prevalent carbapenemase genes (*bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{OXA-48}) in one reaction. The choice of this assay was supported by a recent evaluation demonstrating 100% specificity and sensitivity using a wide variety of strains carrying different carbapenemase genes in vitro.¹¹ Despite an excellent performance, a limitation of this assay is the inability to detect the precise type of carbapenemase. Furthermore, as with all molecular assays, novel unidentified carbapenemase genes may not be detected using this assay.

In total we screened 454 high risk patients and detected six (1.3%) asymptomatic carriers of CPE using this method. Two of the six patients had been hospitalized in Morocco. In both patients the detected isolates were harboring OXA-48, which is known to be endemic in Morocco.¹⁷ Likewise, we detected two KPC producing *K. pneumoniae* isolates in patients hospitalized in Greece and Italy. In both countries KPC-producing *K. pneumoniae* have led to nationwide epidemics. Notably, 3/6 patients were already living outside the Netherlands for several years before hospitalization (Morocco and Greece). The three other patients were hospitalized abroad during a short holiday visit. These patients illustrate the importance of international travel as risk factor for the acquisition and spread of antimicrobial-resistant bacteria, as reviewed by Van der Bij and Pitout.¹⁸ Therefore we think that a screening strategy comparable with the effective

MRSA screening in the Netherlands should also be followed for CPE, in which high risk patients will be kept in contact isolation until the results of screening are available.¹⁹ Furthermore, from the six patients with CPE in our study we did not receive other cultures in which CPE were found. This emphasizes the need for an active surveillance strategy to detect asymptomatic carriers of CPE in order to prevent unnoticed introduction in the hospital.

A recent algorithm for carbapenemase detection suggested a three-step method in which an isolate is screened on a selective medium.²⁰ Thereafter, the Carba NP test (based on *in vitro* hydrolysis of a carbapenem) is performed and subsequently the carbapenemase genes are detected and typed by molecular identification. The results are available within 24 h, which is comparable to our approach. One disadvantage of our screening method is the higher cost associated with molecular diagnostics. However, Tijet et al. recently reported an evaluation of the Carba NP test and confirmed its 100% specificity, but the sensitivity and negative predictive value were 72.5% and 69.2% respectively.²¹ Muroid strains and enzymes with low carbapenemase activity (especially OXA-48) were associated with false-negative results, which is a serious limitation since OXA-48 is emerging globally and is one of the major clinically relevant carbapenemases.¹⁶ This is underlined by the results of our study, in which the majority of detected isolates were actually OXA-48-producing isolates.

In conclusion, this study reports the results of a new screening method to identify CPE in patients within 24 hours. Since early identification and isolation of carriers are key components of an effective infection control strategy in hospitals, this method may be a major improvement in the challenge to reduce the spread of CPE.

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6

EVALUATION OF A NEW REAL-TIME PCR ASSAY (CHECK-DIRECT CPE) FOR RAPID DETECTION OF KPC, OXA-48, VIM AND NDM CARBAPENEMASES USING SPIKED RECTAL SWABS

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Abstract

To prevent the spread of carbapenemase-producing bacteria, a fast and accurate detection of patients carrying these bacteria is extremely important. The Check-Direct CPE assay (Check-Points, Wageningen, The Netherlands) is a new multiplex real-time PCR assay, which has been developed to detect and differentiate between the most prevalent carbapenemase genes encountered in *Enterobacteriaceae* (*bla*_{KPC}, *bla*_{OXA-48}, *bla*_{VIM}, and *bla*_{NDM}) directly from rectal swabs. Evaluation of this assay using 83 non-duplicate isolates demonstrated 100% sensitivity and specificity and the correct identification of the carbapenemase gene(s) present in all carbapenemase-producing isolates. Moreover, the limit of detection (LoD) of the real-time PCR assay in spiked rectal swabs was determined and showed comparable LoDs with the ChromID CARBA agar. With an excellent performance on clinical isolates and spiked rectal swabs, this assay appeared to be an accurate and rapid method to detect *bla*_{KPC}, *bla*_{OXA-48}, *bla*_{VIM}, and *bla*_{NDM} genes directly from a rectal screening swab.

Introduction

The emergence and spread of antimicrobial resistance conferred by the production of beta-lactamases amongst Gram-negative bacteria is a serious problem, in the hospital environment as well as in the community. Due to the emergence of resistance by extended-spectrum beta-lactamases (ESBLs), carbapenem antibiotics are used more often to treat patients with infections caused by Gram-negative bacteria. However, the extensive use of carbapenems is probably the most significant reason for an increasing resistance to this class of beta-lactams by the production of carbapenemases. The major carbapenemases reported worldwide in *Enterobacteriaceae* comprise carbapenemases of Ambler class A (mostly bla_{KPC}), class B (metallo-beta-lactamases; mostly bla_{VIM} and bla_{NDM} and, to a lesser extent, bla_{IMP}), and class D (oxacillinases; mostly, bla_{OXA-48}).^{1,2}

A higher mortality rate is observed in infections caused by carbapenemase-producing *Enterobacteriaceae* and some nonfermenters (such as *Pseudomonas aeruginosa* and *Acinetobacter* spp.) due to inappropriate empirical therapy, which urges for rapid detection.³⁻⁵ Moreover, rapid detection in a hospital setting is crucial for immediate implementation of infection control safety measures to prevent further spread of resistance in the hospital environment.⁶

However, screening for carbapenemase-producing bacteria is time-consuming since it is usually performed using selective agars such as ChromID ESBL, ChromID CARBA (BioMérieux, Marcy-l'Étoile, France), CHROMagar KPC (CHROMagar, Paris, France), or SUPERCARBA medium.⁷ Although the ChromID CARBA, the SUPERCARBA medium, and the less sensitive ChromID ESBL and CHROMagar KPC media are able to detect most carbapenemase producing isolates, the inoculated plates have to be incubated overnight.⁷⁻¹⁰ Subsequently, the presence of carbapenemase producers on the culture medium has to be confirmed by additional testing (e.g., modified Hodge test, gradient strips, or inhibition studies with liquid or solid media), which can take up to another 48 hours.¹¹ Although the use of molecular techniques for confirmation (such as PCR or microarray analysis) or the recently developed biochemical Carba NP assay does shorten the turnaround time to some extent, this approach is not an adequate solution for the rapid detection in patient samples as required.¹¹⁻¹⁵

Recently, several molecular assays have been described detecting carbapenemase genes directly from urine (bla_{KPC})¹⁶, rectal swabs (bla_{KPC})¹⁷, or stool samples (bla_{NDM} and bla_{OXA-48}).^{18,19} These tests, able to detect only 1 carbapenemase gene each (bla_{KPC} , bla_{NDM} , or bla_{OXA-48}), are extremely useful in an outbreak situation caused by a known carbapenemase gene. However, since a variety of genes can be responsible for carbapenemase resistance, these tests will not be appropriate for screening of carbapenemase carriers. Here, we describe the Check-Direct CPE assay (Check-Points, Wageningen, The Netherlands), a new real-time PCR test for rapid and simultaneous detection of the beta-lactamase genes bla_{KPC} , bla_{OXA-48} , bla_{VIM} , and bla_{NDM} that is developed to apply directly on rectal swabs.

Materials and methods

Bacterial isolates

Specificity, sensitivity, and limit of detection (LoD) of this new Check-Direct CPE assay were assessed with 62 unique well-characterized Gram-negative bacterial isolates with proven carbapenemase activity (Table 1), along with 21 non-carbapenemase producers (Table 2). All isolates were suspended in 0.45% saline (NaCl) up to a density of approximately 0.5 McFarland ($\sim 2 \times 10^8$ CFU/ml). In addition, a subset of the carbapenemase-producing isolates (as shown in Table 3) were suspended and used for spiking experiments.

Serial dilutions for spiking experiments

From the 0.5 McFarland suspension of each of the nineteen isolates, seven 10-fold dilutions were made in 0.45% saline (NaCl). A volume of 500 μ L from a total of 100-mL liquid Amies (Sigma transwab, MW&E, Corsham, UK) gathered from rectal swab specimens (collected for routine microbiological diagnostics) from 138 patients was aliquoted. Spiked rectal swabs were made by adding 100 μ L of each dilution to the 500- μ L aliquot. From every spiked sample, 200 μ L was used for DNA extraction. To determine the sensitivity of the ChromID CARBA, 20 μ L of each suspension was used to inoculate the plates and incubated overnight at 37 °C. The number of suspected colonies (pink- or blue-colored according to the manual) was counted the following day. A negative control for culture and the real-time PCR was tested with an aliquot of the pool of rectal swabs without adding a carbapenemase producer. Each experiment was performed in duplicate.

DNA extraction

DNA extraction of 200 μ L of each suspension was performed using the NucliSens EasyMAG (BioMérieux, Marcy-l'Etoile, France), according to the Generic 2.0.1 protocol (on-board lysis and 70- μ L elution volume). Before DNA extraction, 5 μ L of internal control (IC) (Check-Points) was added to each suspension as instructed by the manual. The extraction process took about 1 hour for a maximum of 24 samples.

Real-time PCR

The Check-Direct CPE assay was designed by Check-Points and detects all known variants of *bla*_{VIM} (except *bla*_{VIM-7}), *bla*_{KPC}, *bla*_{NDM}, and *bla*_{OXA-48} including *bla*_{OXA-162,-181,-204}. The real-time PCR was performed on the TaqMan 7500 system (Applied Biosystems, Bleiswijk, The Netherlands) in a 25- μ L Mastermix reaction containing 12.5 μ L of TaqMan Fast Advanced Mastermix (Applied Biosystems), 2.5- μ L CPE solution containing all primers and probes (Check-Points), and 10- μ L DNA template. All probes are based on the molecular beacon technique and labeled with FAM, VIC, TexasRed, or Cy5 for the detection of *bla*_{KPC}, *bla*_{NDM/VIM}, *bla*_{OXA-48}, and the IC, respectively. Since the probes of both *bla*_{NDM} and *bla*_{VIM} are labeled with the fluorochrome VIC, it is not

possible to differentiate between these 2 carbapenemase genes. The real-time PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 60 s. A sample was considered carbapenemase positive when the FAM, VIC, or TexasRed signal crossed the threshold bar, which was adjusted manually in the log-linear range of the PCR, according to the manual (Ct value <45). A sample was inhibited when the Ct value of the IC was >2 higher than the Ct value of the no-template control of the corresponding run. A real-time PCR run took approximately 1 h and 40 min.

Check-MDR CT102

To confirm the results of the Check-Direct CPE assay, all DNA isolates used for the specificity and sensitivity were analyzed with the Check-MDR CT102 assay (Check-Points), using the same purified DNA as tested in the real-time PCR. The Check-MDR CT102 is able to detect most prevalent ESBL (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}) and carbapenemase genes (*bla*_{KPC}, *bla*_{OXA-48}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM}).²⁰

Reproducibility

Performance of the assay (r^2 and slope over 10 log dilutions) was determined with the same serial dilutions as used to establish the LoD. The reproducibility of the assay was determined using DNA of a dilution of each carbapenemase producer with a Ct value of approximately 30. This DNA was tested 10 times on different days and different TaqMan 7500 systems.

TABLE 1 Carbapenemase-producing clinical isolates subjected to the Check-Direct CPE

Ambler class	Carbapenemase type	Isolate	N ^a	Origin ^b	IPM ^c	MEM ^c
Class A	KPC-type (KPC-like, KPC-2)	<i>Enterobacter cloacae</i>	1	UHNN	>16	>16
		<i>Klebsiella pneumoniae</i>	1	HPA	>16	>16
			5	MGD	>16	>16
			6	UHNN	>16	>16
			3	RS	8 to >16	>16
Class B	NDM-type (NDM-like, NDM-1)	<i>Acinetobacter baumannii</i>	1	MGD	>16	4
		<i>Acinetobacter pittii</i>	1	MGD	8	>16
		<i>Enterobacter cloacae</i>	1	MGD	>16	>16
		<i>Escherichia coli</i>	2	MGD	>16	>16
			2	UHNN	8 to >16	>16
		<i>Klebsiella pneumoniae</i>	1	HPA	>16	>16
			2	MGD	>16	>16
			1	UHNN	>16	>16
		<i>Morganella morganii</i>	1	MGD	>16	>16
		<i>Providencia rettgeri</i>	1	MGD	8	>16
		<i>Klebsiella pneumoniae</i>	1	UHNN	>16	>16
			1	MGD	8	>16
		<i>Aeromonas caviae</i>	1	MGD	>16	>16
		<i>Citrobacter braakii</i>	1	MGD	>16	>16
		<i>Enterobacter cloacae</i>	1	MGD	>16	>16
VIM- + OXA-48-type	VIM-type (VIM-like, VIM-1, VIM-2, VIM-4, VIM-27, VIM-31)	<i>Klebsiella oxytoca</i>	1	MGD	>16	>16
		<i>Klebsiella pneumoniae</i>	2	UHNN	8	>16
			1	HPA	>16	>16
		<i>Pseudomonas aeruginosa</i>	3	MGD	>16	>16
			3	UHNN	>16	>16
		<i>Citrobacter freundii</i>	1	MGD	>16	>16
			1	MGD	>16	>16

TABLE 1 (continued)

Ambler class	Carbapenemase type	Isolate	N ^a	Origin ^b	IPM ^c	MEM ^c
Class D	OXA-48-type	<i>Citrobacter freundii</i>	1	MGD	>16	1
		<i>Enterobacter aerogenes</i>	1	MGD	>16	4
		<i>Enterobacter cloacae</i>	1	MGD	8	4
			1	RS	>16	4
		<i>Escherichia coli</i>	1	HPA	>16	>16
			2	MGD	2	1-2
			1	RS	ND	4
		<i>Klebsiella oxytoca</i>	1	MGD	2	1
		<i>Klebsiella pneumoniae</i>	1	HPA	4	2
			2	MS	8 to >16	>16
			3	MGD	2 to >16	1 to >16
			2	RS	8 to >16	4 to >16

"-like" indicates that the gene was detected by PCR but not sequenced
^a N= number of isolates (total 62)

^b Origin of isolates: UHNH, University Hospital of North Norway, Tromsø, Norway; HPA, Health Protection Agency, NCTC reference strains; MGD, CHU Mont-Godinne-Dinant, Louvain, Belgium; RS, Rijnstate Hospital, Velp, The Netherlands; MS, outbreak strains Maastricht Hospital, Rotterdam, The Netherlands

^c MIC determined by Vitek2 (BioMérieux, Marcy l'Etoile, France); IPM, Imipenem; MEM, Meropenem

TABLE 2 Non-carbapenemase-producing clinical isolates subjected to the Check-Direct CPE assay

Isolate	Origin	Notes
<i>Enterococcus faecalis</i>	ATCC 29212	
<i>Staphylococcus aureus</i>	NCTC 10442	MRSA
<i>Staphylococcus aureus</i>	ATCC 25923	
CoNS ^a	Laboratory strain	
<i>Salmonella typhimurium</i>	Laboratory strain	
<i>Escherichia coli</i>	ATCC 25922	
<i>Campylobacter jejuni</i>	NCTC 11351	
<i>Klebsiella pneumoniae</i>	Laboratory strain	
<i>Klebsiella oxytoca</i>	ATCC 700324	
<i>Enterococcus casseliflavus</i>	ATCC 700327	
<i>Campylobacter jejuni</i>	ATCC 29428	
<i>Klebsiella pneumoniae</i>	ATCC 700603	ESBL SHV
<i>Enterococcus faecalis</i>	ATCC 51299	VRE
<i>Escherichia coli</i>	Laboratory strain	
<i>Pseudomonas aeruginosa</i>	Laboratory strain	Meropenem resistant
<i>Escherichia coli</i>	Laboratory strain	ESBL CTX-M-9, TEM WT
<i>Enterobacter aerogenes</i>	Laboratory strain	
<i>Citrobacter freundii</i>	Laboratory strain	
<i>Pseudomonas aeruginosa</i>	Laboratory strain	Meropenem resistant, AmpC CMY-2, TEM WT
<i>Klebsiella pneumoniae</i>	Laboratory strain	ESBL CTX-M-1, SHV WT, TEM WT
<i>Klebsiella pneumoniae</i>	Laboratory strain	ESBL CTX-M-1, SHV WT, TEM WT

^a CoNS, Coagulase negative Staphylococci

Results

Analytical specificity

The sequences of the primers and probes were tested for specificity using the online BLAST search program of NCBI (<http://blast.ncbi.nlm.nih.gov/>). No significant matches other than those for the included *bla*_{KPC}, *bla*_{OXA-48}, *bla*_{VIM}, and *bla*_{NDM} carbapenemase genes were found as stated by the manufacturer. In addition, the performance of the Check-Direct CPE assay was tested using 62 carbapenemase-producing bacteria (comprising 16 *bla*_{KPC}, 13 *bla*_{NDM}, 1 *bla*_{NDM+OXA-181}, 17 *bla*_{OXA-48}, 14 *bla*_{VIM}, and 1 *bla*_{VIM+OXA-48}). All isolates with known carbapenemase genes were detected using DNA isolated from a 0.5 McFarland suspension and a threshold of 0.01. The isolates were all correctly detected as KPC, NDM/VIM, or OXA-48 (100% sensitivity) with a Ct

value ranging from 17.4 to 18.1 (mean: 18.0), 21.3 to 26.0 (mean: 24.0), 14.7 to 18.4 (mean: 15.4), and 19.1 to 23.1 (mean: 20.6) for KPC, NDM, OXA-48, and VIM, respectively. The assay was able to detect both carbapenemase genes in the isolates containing 2 carbapenemase genes ($bla_{NDM+OXA-181}$ and $bla_{VIM+OXA-48}$). Furthermore, negative results (no amplification signal) were correctly obtained for the 21 carbapenemase-negative isolates, comprising 4 ESBL producers (1 bla_{SHV} , 1 bla_{CTX-M9} , 2 $bla_{CTX-M15}$), 2 meropenem resistant *P. aeruginosa* (1 bla_{CMY-2} , no resistance mechanism detected in the second), 1 vancomycin-resistant *Enterococcus faecalis* (VRE, ATCC51299), 1 methicillin-resistant *Staphylococcus aureus* (MRSA, NCTC10442), and 13 other miscellaneous isolates representing the flora from the gastrointestinal tract (100% specificity). These results were confirmed using the Check-MDR CT102 (data not shown). All results were available within 3 h (1 h DNA extraction plus 1 h and 40 min real-time PCR).

LoD in rectal swabs

The LoD of the Check-Direct CPE assay, directly from rectal swabs, was determined with serial dilutions of 19 different carbapenemase-producing isolates spiked in a pool of rectal swabs as described in the Materials and methods section. The LoD of the assay varied from 200–2000 CFU/mL to 2×10^4 to 2×10^5 CFU/mL, corresponding to 1–10 CFU/PCR and 100–1000 CFU/PCR, as shown in Table 3. In the rectal swabs that remained carbapenemase negative, no PCR inhibition was seen. LoD for the ChromID CARBA was determined by using the same spiked samples and varied from 200–2000 CFU/mL to 2×10^7 to 2×10^8 CFU/mL (Table 3). The negative control (aliquot of the pool of rectal swabs without adding a carbapenemase producer) remained negative in both the Check-Direct CPE assay and the ChromID CARBA medium.

Dynamic range of the Check-Direct CPE assay

The detection was linear over 10 log dilutions with a mean r^2 of 0.991, 0.998, 0.987, and 0.993 and a slope of -3.91 , -3.58 , -3.55 , and -3.90 for bla_{KPC} , bla_{OXA-48} , bla_{VIM} , and bla_{NDM} , respectively. The reproducibility of the assay, using a dilution of each carbapenemase producer with a Ct value of approximately 30, showed that the Check-Direct CPE assay is highly stable and precise. After 10 repeats, the range between minimum and maximum, mean Ct value, SD, and 95% confidence interval (CI) for each carbapenemase were as follows: bla_{VIM} (range: 2.47, mean: 30.7, SD: 0.85, CI: ± 0.61), bla_{OXA-48} (range: 2.15, mean: 28.8, SD: 0.70, CI: ± 0.50), bla_{NDM} (range: 3.48, mean: 30.4, SD: 1.16, CI: ± 0.83), and bla_{KPC} (range: 3.87, mean: 30.8, SD: 1.26, CI: ± 0.90).

TABLE 3 LoD determination using spiked rectal swabs

Carbapenemase type	Isolate	Origin ^a	IPM ^b	MEM ^b	LoD in spiked rectal swab			
					ChromID Carba		Check-Direct CPE	
					CFU/ml ^c	CFU/plate ^d	CFU/ml ^c	CFU/PCR ^e
VIM-1	<i>Klebsiella pneumoniae</i>	UHNH	8	>16	2x10 ³ -2x10 ⁴	7-70	200-2000	1-10
VIM-31	<i>Enterobacter cloacae</i>	MGD	>16	>16	2x10 ⁵ -2x10 ⁶	700 - 7000	2x10 ³ -2x10 ⁴	10-100
VIM-1	<i>Klebsiella oxytoca</i>	MGD	>16	>16	2x10 ³ -2x10 ⁴	7-70	2x10 ³ -2x10 ⁴	10-100
VIM-27	<i>Klebsiella pneumoniae</i>	MGD	>16	>16	2x10 ³ -2x10 ⁴	7-70	2x10 ³ -2x10 ⁴	10-100
VIM-4	<i>Pseudomonas aeruginosa</i>	MGD	>16	>16	2x10 ⁶ -2x10 ⁷	7x10 ³ -7x10 ⁴	2x10 ⁴ -2x10 ⁵	100-1000
OXA-48	<i>Klebsiella pneumoniae</i>	MS	8	>16	200-2000	0.7-7	2x10 ³ -2x10 ⁴	10-100
OXA-48	<i>Klebsiella pneumoniae</i>	HPA	4	2	2x10 ⁶ -2x10 ⁷	7x10 ³ -7x10 ⁴	2x10 ³ -2x10 ⁴	10-100
OXA-48	<i>Enterobacter aerogenes</i>	MGD	>16	4	2x10 ⁷ -2x10 ⁸	7x10 ⁴ -7x10 ⁵	200-2000	1-10
OXA-48	<i>Klebsiella pneumoniae</i>	MGD	>16	>16	200-2000	0.7-7	2x10 ³ -2x10 ⁴	10-100
NDM like	<i>Escherichia coli</i>	UHNH	>16	>16	200-2000	0.7-7	2x10 ³ -2x10 ⁴	10-100
NDM like	<i>Klebsiella pneumoniae</i>	HPA	>16	>16	2x10 ³ -2x10 ⁴	7-70	2x10 ³ -2x10 ⁴	10-100
NDM-1	<i>Klebsiella pneumoniae</i>	MGD	>16	>16	2x10 ³ -2x10 ⁴	7-70	2x10 ³ -2x10 ⁴	10-100
NDM-1	<i>Klebsiella pneumoniae</i>	MGD	>16	>16	200-2000	0.7-7	2x10 ³ -2x10 ⁴	10-100
NDM like	<i>Providencia rettgeri</i>	MGD	8	>16	2x10 ⁴ -2x10 ⁵	70-700	2x10 ³ -2x10 ⁴	10-100
KPC like	<i>Enterobacter asburiae</i>	UHNH	>16	>16	200-2000	0.7-7	2x10 ³ -2x10 ⁴	10-100
KPC like	<i>Klebsiella pneumoniae</i>	MGD	>16	>16	200-2000	0.7-7	2x10 ³ -2x10 ⁴	10-100
KPC like	<i>Klebsiella pneumoniae</i>	MGD	>16	>16	200-2000	0.7-7	2x10 ³ -2x10 ⁴	10-100
KPC-2 like	<i>Klebsiella pneumoniae</i>	MGD	>16	>16	200-2000	0.7-7	2x10 ³ -2x10 ⁴	10-100
KPC like	<i>Klebsiella pneumoniae</i>	MGD	>16	>16	200-2000	0.7-7	200-2000	1-10

^a Origin of isolates. UHNN, University Hospital of North Norway, Tromsø, Norway; HPA, Health Protection Agency, NCTC reference strains; MGD, CHU Mont-Godinne-Dinant, Louvain, Belgium; RS, Rijnstate Hospital, Velp, The Netherlands; MS, outbreak strains Maasstad Hospital, Rotterdam, The Netherlands

^b Minimal Inhibitory Concentration (mg/l) MIC determined by Vitek2 (BioMérieux, Marcy l'Etoile, France); IPM, Imipenem; MEM, Meropenem

^c the LoD (limit of detection) is calculated using tenfold dilutions starting with 0.5 McFarland (~2x10⁸ CFU)

^d 20 µl of the spiked suspension is inoculated on the ChromID carba. CFU/plate is the amount of CFU calculated from the original 0.5 McFarland suspension

10 µl of DNA isolate is used in the Check-Direct CPE. CFU/PCR is the amount of CFU calculated from the original 0.5 McFarland suspension

Discussion

The most prevalent carbapenemases worldwide are IMP, VIM, OXA-48, NDM, and KPC, with the latter 4 being mostly distributed throughout Europe.^{2,21} Early identification in clinical infections and in patients colonized with carbapenemase-producing bacteria, both *Enterobacteriaceae* and nonfermenters (such as *P. aeruginosa* and *Acinetobacter* spp.), is important to prevent the spread of carbapenemase producers. This study describes the first evaluation of the Check-Direct CPE assay, a new real-time PCR test developed to detect the most prevalent and clinically important carbapenemase genes *bla*_{KPC}, *bla*_{OXA-48}, *bla*_{VIM}, and *bla*_{NDM} directly from rectal swabs within 3 h.

To evaluate the Check-Direct CPE assay, a total of 83 unique isolates were analyzed, comprising 62 different earlier molecularly characterized carbapenemase producers and 21 various non-carbapenemase producers. The results of the Check-Direct CPE assay showed 100% agreement with the previously defined genotypes and the results obtained with the Check-MDR CT102. For the evaluation of the Check-Direct CPE assay on rectal swabs, the LoD was determined with spiked samples, using 19 different isolates. The LoD varied within most groups of tested carbapenemase genes. The lowest LoD determined using *bla*_{OXA-48} was 1–10 CFU/PCR (*Enterobacter aerogenes*), while the remaining 3 isolates (all *Klebsiella pneumoniae*) gained an LoD of 10–100 CFU/PCR (Table 3). Naas *et al.* analyzed the LoD of an in-house developed real-time PCR for *bla*_{OXA-48} in spiked fecal suspensions and found 1–5 CFU/PCR using *Escherichia coli*, which is about 20 times lower than the (highest) LoD of 100 CFU/PCR *bla*_{OXA-48} found with the Check-Direct CPE assay.¹⁸ Naas *et al.* also analyzed the LoD in spiked fecal suspensions of another in-house developed real-time PCR, able to detect *bla*_{NDM}, and found an LoD of less than 1 CFU/PCR. This is about 10–100 times lower than the LoD of 10–100 CFU/PCR as determined by the Check-Direct CPE assay.¹⁹ The in-house real-time PCRs as developed by Naas *et al.* were both monoplex reactions, which were detected by a TaqMan probe labeled with the most efficient reporter: FAM. Since the Check-Direct CPE assay is a complex multiplex assay, which is able to differentiate between the carbapenemase genes *bla*_{KPC} (FAM label), *bla*_{OXA-48} (Texas Red label), and *bla*_{NDM/VIM} (both VIC label), the sensitivity can be slightly reduced. In addition, *bla*_{NDM} and *bla*_{OXA-48} are detected on the TaqMan 7500 system using the less efficient reporters VIC and TexasRed, respectively, which possibly influence the difference in LoDs. For

the LoD of bla_{VIM} and bla_{KPC} , no comparison with other studies is possible, since no molecular assays determining the LoD of these carbapenemase genes in (spiked) rectal swabs or stool has been published yet. Although the analytical sensitivity of the Check-Direct CPE assay is not as good as the in-house developed multiplex real-time PCRs as described previously, the capability of the Check-Direct CPE assay to detect the 4 major carbapenemase genes simultaneously and directly differentiate between bla_{KPC} , bla_{OXA-48} , and $bla_{NDM/VIM}$ is an important feature, especially in situations where screening of carbapenemase carriers is needed and in outbreak situations.

Nowadays screening for carbapenemase-producing bacteria is most often performed with a selective medium. Therefore, in this study, we compared the sensitivity of the Check-Direct CPE assay to the ChromID CARBA medium, which has been selected because of its reported high sensitivity and specificity.^{10,22} As illustrated in Table 3, LoD (CFU/mL) of the Check-Direct CPE assay shows comparable sensitivity with the ChromID CARBA concerning bla_{VIM} and bla_{NDM} . Although ChromID CARBA has a lower LoD for 2 of the bla_{OXA-48} producing *K. pneumoniae* isolates, it clearly shows the lack of correct detection when lower MICs are present. This is in concordance with the results of the study of Vrioni *et al.*, which earlier showed a high LoD (1.1×10^7 CFU/mL) when the ChromID CARBA was tested with carbapenemase producers harboring low MICs.¹⁰ Finally, LoD of bla_{KPC} determined with the Check-Direct CPE assay was approximately 10 times lower than the LoD of the ChromID CARBA. A study of Wilkinson *et al.* earlier showed good detection of bla_{KPC} using ChromID CARBA, since this medium was able to detect 100% (12/12) of bla_{KPC} carbapenemase producers with low inoculums, whereas isolates producing bla_{VIM} , bla_{IMP} , bla_{OXA-48} , and bla_{NDM} with low inoculums were correctly detected in 83.3% (5/6), 88.9% (8/9), 66.7% (10/15), and 94.3% (83/88), respectively.²² Overall, in comparison with the ChromID CARBA medium, directly from rectal swabs, the Check-Direct CPE assay showed comparable results in detecting carbapenemase genes bla_{OXA-48} , bla_{VIM} , and bla_{NDM} and acceptable results for bla_{KPC} . However, when carbapenemase producers with low MICs are subjected for detection, the Check-Direct CPE assay is superior over the ChromID CARBA medium.

The turnaround time is clearly in favor of the Check-Direct CPE assay and other recently published molecular methods (all approximately 3–4 h) over any other selective agar because absence of carbapenemase producers cannot be established earlier than the next day since it will always need an overnight incubation step. Moreover, carbapenemase suspected isolates should be confirmed since ESBLs or AmpC beta-lactamase producers can give false-positive results.¹¹ This confirmation step can take up to another 48 h, depending on the test used. Chromogenic media can also generate false-negative results, which might be explained by carbapenemases with very low MICs.^{7,11}

Kochar *et al.* demonstrated that a good infection control program, consisting of improved hand hygiene and routine rectal surveillance cultures, decreased the spread of bla_{KPC} in an intensive care unit significantly.⁶ Using the Check-Direct CPE assay, instead of routine rectal surveillance cultures, infection control programs can become more efficient.

Although the Check-Direct CPE assay has a rapid and excellent performance, it should be emphasized that this assay is only able to identify the 4 major carbapenemase genes *bla*_{KPC}, *bla*_{OXA-48}, *bla*_{VIM} and *bla*_{NDM}. As in all molecular assays, other or newly discovered carbapenemase genes will not be detected.

This study has several limitations. First, not all known variants of the 4 carbapenemase genes were included in this study. Therefore, we were not able to evaluate the correct detection of all variants of carbapenemase genes. Second, we used spiked rectal swab samples to investigate the performance of the Check-Direct CPE assay on this specimen type. Since the prevalence of carbapenemase producers in The Netherlands is very low, a study of direct detection in rectal swabs from patients is almost impossible. Although spiked rectal swabs are not real clinical samples, it certainly is the most appropriate way to mimic the performance of the Check-Direct CPE assay in this specimen type. It is important to know that in our routine culture screening, we did find 4 different rectal swabs with a carbapenemase producer (3 *bla*_{OXA-48} and 1 *bla*_{KPC}). These rectal swabs were set aside and tested according to the procedure as used in this study and all gained a clear positive PCR result (data not shown). Although these results of direct detection in rectal swabs from patients are very promising, further studies in patients from endemic countries are necessary.

In conclusion, this new multiplex real-time PCR assay appeared to be an accurate and stable method to detect the most prevalent and clinically important carbapenemase genes *bla*_{VIM}, *bla*_{OXA-48}, *bla*_{NDM} and *bla*_{KPC}. This sensitive method could be applied directly on rectal samples and is able to differentiate between the carbapenemase genes *bla*_{KPC}, *bla*_{OXA-48} and *bla*_{VIM/NDM} obtaining results within 3 h. The assay looks promising as a carbapenemase screening method and can be an important improvement in infection control programs.

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7

GENERAL DISCUSSION

Introduction

The discovery of antibiotics is considered one of the most important findings of the 20th century, making it possible to cure life-threatening bacterial infections. Due to the proven success of antibiotics, they were extensively used for treatment of all kinds of bacterial infections worldwide. Moreover, introduction of antibiotics in the farming industry for disease prevention and growth promotion, was responsible for another rise in the use of antibiotics. As a consequence of the increasing use of antibiotics, introduction of antimicrobial resistance followed rapidly.

However, despite the increasing use of antibiotics and subsequent rise in antimicrobial resistance, no new drugs have been developed in the past 27 years.¹ As a result, antibiotic resistance in bacteria is now emerging worldwide and described in the 'Global report on the surveillance of antimicrobial resistance' by the World Health Organization (WHO) in April 2014.²

To prevent further dissemination and global spread of antimicrobial resistance, rapid and correct identification of highly resistant microorganisms (HRMOs) is mandatory in order to subsequently apply optimal antibiotic therapy and/or implement infection control measures as rapid as possible.^{3,4} Therefore, different guidelines have been developed describing recommendations on this matter.⁵⁻⁷ However, these guidelines recommend to screen for HRMOs using selective media, followed by confirmation using phenotypic methods or molecular methods (if available). Unfortunately, the phenotypic methods are time-consuming, can generate incorrect results and mostly cannot generate data that can be used for epidemiological purposes.

The aim of this thesis was to study different strategies and methods to improve the laboratory detection of HRMOs and decrease the time to result. Therefore, a variety of molecular methods have been used for the detection of the clinically most important resistance markers of methicillin resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamases (ESBL) and carbapenemase producing *Enterobacteriaceae* (CPE).

Molecular confirmation methods

According to the guidelines on the laboratory detection of HRMOs, selective media is the method of choice to screen for the presence of either MRSA, ESBL or CPE. When isolates suspected to be MRSA, ESBL or CPE are identified, confirmation should always be performed since none of the selective media shows 100% specificity. Therefore, either phenotypic or genotypic methods can be used. However, besides the confirmation of MRSA, for which a rapid and reliable latex agglutination test has been developed⁸, the phenotypic confirmation methods are time consuming and have shown to produce false-positive results in case of overproduction of other enzymes or mechanisms. Therefore, confirmation by molecular methods is necessary.

For the confirmation of MRSA and CPE, the assays that have been evaluated as (part of the) screening method directly on DNA isolated from rectal swabs have also shown to be very reliable

and useful as confirmation method. In **chapter 4**, the performance of the molecular assays for the detection of MRSA have been evaluated demonstrating very high sensitivity on 104 assorted MRSA isolates. For the confirmation of CPE, the Check-Direct CPE assay has been evaluated using 83 non-duplicate isolates. This method was able to detect all 62 unique carbapenemase producers correctly and no cross-reactivity was observed with any of the 21 non-carbapenemase producers (**chapter 6**). Finally, the performance of the Check-MDR Carba assay (**chapter 5**) has been evaluated by Cuzon *et al.* using 183 well-characterized Gram-negative rods, showing a very accurate identification (sensitivities and specificities of 100%).⁹

Regarding ESBL, no active surveillance is performed in The Netherlands, since the prevalence in the community has shown to be around 9%.^{10,11} However, when in an hospital an unexpected ESBL producer is identified, the patient has to be nursed in contact isolation and targeted ESBL screening is performed when necessary.³ So far, the only molecular assay that has been developed for the detection of ESBL directly on DNA isolated from a rectal swab is the HyplexID ESBL assay (AmplexDiagnostics GmbH, Gars-Bahnhof, Germany). However, to the best of our knowledge, this PCR-ELISA based assay has not yet been evaluated using rectal swabs. Therefore, as described in the recommendations, targeted ESBL screening should be performed using selective media with subsequent confirmation using phenotypic or genotypic methods.⁷ Here, the genotypic methods are favorable since their short time-to-result, compared to phenotypic methods. However, the genotypic methods as recommended by the guidelines - PCR and ESBL gene sequencing or a DNA microarray - need specialized systems and highly trained personnel.

In **chapter 2**, the evaluation of a novel, easy-to-use ligation-mediated real-time PCR assay (Check-MDR ESBL assay) for the detection of ESBL is described. The performance of this assay is compared with the performance of a widely used phenotypic ESBL confirmation method, the combined disk test (CDT). The comparison consisted of 172 isolates suspected to be ESBL positive according to susceptibility testing by VITEK2. The Check-MDR ESBL assay, designed to detect the most prevalent ESBL genes (TEM-E104K, TEM-R164S, SHV-G238S, CTX-M1-group, CTX-M2-group and CTX-M9-group), demonstrated a sensitivity and specificity of 99.0% and 92.2% respectively, using CDT as standard. In this study, we found 6 isolates to be positive using the CDT method, while these remained negative in the Check-MDR ESBL assay. PCR and sequencing and the Check-MDR CT103 microarray confirmed the correct results of the Check-MDR ESBL assay regarding these 6 isolates, since no other TEM, SHV or CTX-M ESBLs have been detected.

The high sensitivity and specificity of the assay could be confirmed by a recent evaluation by Willemssen *et al.*, showing sensitivity and specificity of 98.9% (350/354) and 100% (133/133) respectively.¹² The sensitivity in the particular study was calculated using the initial results obtained with the Check-MDR ESBL assay, in which 4 isolates were determined false-negative. One of these isolates, however, was detected correctly positive after re-testing. The remaining 3 false-negative isolates contained ESBL-genes for which no primers or probes are included in the assay (TEM-G238S (n=2) and SHV-G238A+E240K (n=1)).

Using a Dutch multicentre study developed to determine the ESBL-genes present in the Netherlands, it was calculated that 3.1% (17/551) of all detected ESBL isolates could not have been detected using the Check-MDR ESBL assay.¹³ The majority of these isolates (10/17) were identified as ESBL TEM-G238S. In addition, ESBL genes found in the particular study that cannot be detected using the Check-MDR ESBL assay comprise SHV-31 (L35Q, E89K, A243G; n=2) CTX-M8/25 (n=2), GES (n=2) and PER (n=1). Although a high percentage of ESBL producing isolates (96.9%) will be detected using the Check-MDR ESBL assay, both Willemsen *et al.* and Voets *et al.* have shown that ESBL TEM-G238S is the main gene that cannot be detected. Therefore, the addition of primers/probes to identify this ESBL variant seems mandatory.

In the study described in **chapter 2**, six isolates were positive using the CDT method, but tested negative in the Check-MDR ESBL assay. Although TEM-G238S has shown to be the main variant that cannot be detected using the Check-MDR ESBL assay, discrepancy testing using PCR and sequencing and the Check-MDR CT103 microarray excluded the possibility of false-negative results due to this or any other TEM, SHV or CTX-M variant. However, although the evaluation was concentrated on the correct detection of the most prevalent ESBL genes (i.e. TEM, SHV and CTX-M), we cannot exclude that the CDT did correctly identify ESBL production mediated by other, more rare ESBL genes such as VEB-, PER- or GES-ESBL. In order to obtain definitive results, 5 out of the 6 isolates could be tested using the beta-version of a new DNA micro-array, the Check-MDR CT103XL, that includes detection of GES-, PER-, VEB-, and BEL-ESBL. No additional ESBL-genes were found in the 5 isolates, meaning that these isolates were truly determined false-positive by CDT in our study. False-positive results obtained by phenotypic methods for the detection of ESBL has also been demonstrated by others.^{14,15} Therefore, compared to the phenotypic CDT method, the evaluated molecular assay showed to be a reliable, easy-to-use and more rapid method to detect ESBL.

As described in the guidelines, DNA microarray assays can also be used for the confirmation of ESBLs. Although these assays need special equipment and highly trained personnel, these methods have the possibility to generate epidemiological data, which is an important feature that can be extremely useful for hygiene and infection control practitioners. In **chapter 3**, a commercially available DNA microarray (the Check-ESBL assay), that has earlier shown to be able to correctly identify and specify the group of ESBL present¹⁶, was used to study the distribution of ESBL genes in our region.

The distribution of ESBL genes found in our study was comparable with the distribution of ESBL genes nation-wide, with CTX-M1 and CTX-M9 being most often detected, followed by SHV, TEM and CTX-M2 group ESBL.¹³ Moreover, the findings of both our study and the nation-wide study are conform the global spread of CTX-M ESBLs, where in general, CTX-M15 and CTX-M14, belonging to respectively CTX-M1 group and CTX-M 9 group, are most often detected in clinical isolates.^{17,18} Only regarding ESBL producing *E. cloacae* our results differ from the results of Voets *et al.*¹³ Here, the proportion of ESBL genes found in *E. cloacae* is CTX-M1 > SHV > CTX-M9, while in our study the proportion was CTX-M9 > SHV > CTX-M1. Although

we discussed that this might be due to the fact that CTX-M9 is associated with *E. cloacae*, this is not supported by the multicentre study. This increased prevalence of *E. cloacae* harboring CTX-M9 can also not be explained by the fact that *E. cloacae* has been found significantly more in hospitalized patients, since Voets *et al.* also showed a high number of *E. cloacae* in this group of patients compared to outpatients. A plausible explanation might be that there was simply an increased prevalence of CTX-M9 ESBL producing *E. cloacae* in our region at the time of the study.

Molecular detection following broth enrichment

Molecular assays that can be used for the confirmation of MRSA and CPE have shown excellent performance testing a high number of different isolates. As part of this thesis, these molecular assays have been applied in a new approach for the detection of resistance genes. Here, although the guideline does not describe any recommendations on the use of molecular assays following broth enrichment⁷, the molecular assays are tested on DNA extracted from enriched broths in order to decrease the time-to-result.

For detection of MRSA, the Dutch guideline recommends to differentiate between methods that should be used in case of urgency or no urgency. When urgency testing has to be performed, the clinical specimen is tested by either direct molecular testing (point of care testing (POCT)) or by culturing the clinical specimen directly on a specific MRSA screening agar. The added value of this urgency testing was clearly shown in a Dutch multi-centre study, showing a reduction in the number of isolation days of up to 60.4%, regardless of the method used.¹⁹ However, sensitivity of these assays reached a maximum of only 85.7% when compared to conventional cultures that included overnight broth enrichment. Therefore, the guideline recommends to subsequently test all urgency samples with conventional methods consisting of broth enrichment (18-24 hours) followed by culture on an MRSA screening agar, that has to be incubated 42-48 hours and interpreted after both 18-24 hours and 42-48 hours. In total, the additional recommended conventional methods will need at least 72 hours (3 days) to gain either a negative or a positive result.

To maintain the short time to result as observed in urgency testing, and overcome the issue of sensitivity because of the lack of overnight broth enrichment, we introduced a molecular screening approach that indeed does include this overnight incubation step.

The study as described in **chapter 4**, shows the results of a 2-year period using the screening approach in which broth enrichment is followed by a combination of two real-time PCR assays. Here, 13.387 samples were analyzed, of which 95.2% could be reported as negative within 24 hours. Moreover, since our approach consists of (in-house) real-time PCR assays targeting the *S. aureus* specific gene SA442 and the *mecA* and *mecC* gene with subsequent confirmation using a commercially available assay that targets the *SCCmec-orfX* junction, results of highly suspected MRSA samples can also be obtained within 24 hours. This is a reduction of at least

48 hours in time to result when compared to the approach (urgency testing and subsequent confirmation using conventional methods) that is recommended by the Dutch guideline. Because reliable, definitive results are obtained earlier, consequential infection control measures can be implemented more rapidly. This is of particular importance in case of putative transmission, possibly leading to outbreaks. As shortly touched in **chapter 4**, several outbreaks occurred in both hospital and nursing homes in 2013. Our rapid and high throughput approach will most likely be key element in managing such outbreaks, since infection control practitioners are able to create an excellent overview on the possible transmission of MRSA within 24 hours.

The current Dutch guideline states that there is still too limited available evidence to recommend the use of broth enrichment in combination with molecular detection methods. To date, several studies have been published addressing this issue. Three studies compared culture with different real-time PCR assays, targeting different *S. aureus* specific genes and the *mecA* gene, in which both are preceded with overnight broth enrichment. All assays were able to correctly identify MRSA from the broths using the real-time PCR assays, in the samples that have been identified MRA positive by culture (sensitivity 100%).²⁰⁻²² Kerremans *et al.* performed a likewise study, although they used the BD GeneOhm MRSA assay after broth enrichment. Nevertheless, they showed good performance of this method, since it showed a sensitivity of 94%.²³ Hence, combining broth enrichment with molecular methods has shown to be an effective tool for the detection of MRSA.

The study as described in **chapter 4**, showing the clinical application of a likewise approach, clearly shows the added value of combining broth enrichment and molecular methods. With the current knowledge, there seems to be no more reason not to include recommendations on the detection of MRSA using broth enrichment in combination with molecular methods in a revision of the current guidelines.

Because of the good performance of broth enrichment followed by molecular detection of MRSA, a comparable screening approach was developed for the detection of CPE. In **chapter 5**, we described the clinical application of a new screening method, developed for the rapid detection of CPE in putative carriers. This method consists of broth enrichment followed by genotypic detection using a ligation-mediated real-time PCR assay (Check-MDR Carba) that can accurately detect variants of the most prevalent and clinically important carbapenemase genes *bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM} and *bla*_{OXA-48} and determine the presence or absence of isolates carrying these genes in the overnight incubated broths within 4.5 hours.^{9,24}

For the initial overnight enrichment, two different tryptic soya broths (TSB), supplemented with either ertapenem (0.25 µg/ml) or ceftazidime (2 µg/ml) are used. Although the recommended strategy of the Center for Disease Control and prevention (CDC) is also using a TSB supplemented with ertapenem, they recommend a 2 µg/ml concentration.⁵ However, since various studies have shown carbapenemase producing isolates with ertapenem MIC values of <2 µg/ml^{25,26}, such isolates would not be detected using the CDC method. Besides, in our

approach, a TSB broth supplemented with ceftazidime is added. Since a high number of CPE isolates co-produce an ESBL, the addition of this broth will increase the sensitivity of the method.

Following our screening program for 14 months, we found samples from 6 out of 454 (1.3%) patients to be positive in the Check-MDR Carba assay. Each PCR positive sample was cultured and one or more CPE isolates could be identified, confirming the PCR positive results. In four of the patients an OXA-48 carbapenemase was detected, while in the remaining two patients a KPC producer was detected. Although the numbers are small, OXA-48 was most prevalent detected, as it also is nationwide, according to the National Surveillance of CPE organized by the national reference laboratory RIVM (http://www.rivm.nl/Documenten_en_publicaties/Algemeen_Actueel/Uitgaven/Infectieziekten_Bulletin/Jaargang_25_2014, Bilthoven, the Netherlands).

The approach for the detection of CPE as described in **chapter 5** has been introduced in the beginning of March 2012. Since this introduction, few groups have proposed other strategies for rapid detection of CPE. Comparable to the recommendations described in the Dutch guidelines, these strategies use an initial screening with selective media, followed by a confirmation method. The confirmation methods used in the proposed strategies involve the hydrolysis of carbapenem antibiotics, e.g. the Carba NP test or Maldi-TOF MS.^{27–29} These methods are developed to detect isolates capable of hydrolyzing carbapenem, rather than detecting the presence of specific carbapenemase genes. The proposed combination of selective media with a rapid hydrolysis assay, is able to obtain definitive results within 24 hours, which is equal to our approach. However, since our approach includes broth enrichment, increased analytical sensitivity is obtained compared to direct cultivation on selective media as used in the proposed strategies. This is determined by spiking rectal swabs with serial dilutions of 5 different CPE isolates. The combination of broth enrichment with subsequent molecular testing showed a better analytical sensitivity in 4 of the tested isolates. In one isolate (VIM-producing *E. cloacae*), the analytical sensitivity of the selective medium showed better results than our approach (unpublished data). Although only 5 different isolates have been tested, these results suggest that our approach will be able to detect more CPE from rectal swab samples, compared to direct cultivation on selective media. To confirm this finding, further studies are needed.

Direct molecular testing

The main disadvantage of the approaches that include broth enrichment is the time required for overnight incubation. A significant reduction in time to result would be detection of resistance genes directly from rectal swabs. For MRSA, as discussed earlier, POCT assays have been developed generating results within 2 hours, but showing lack of sensitivity in comparison with standard conventional methods. For CPE, real-time PCR assays that can be applied directly on rectal swabs have been developed. Although the initial assays have been developed for the detection of a single variant, to be used in outbreak situations^{30–33}, other, more extensive assays

have also been evaluated.^{34–36}

In this light, the performance of a new real-time PCR assay (Check-Direct CPE), that has been developed for rapid detection and differentiation of the most prevalent carbapenemase genes (*bla*_{KPC}, *bla*_{VIM}, *bla*_{NDM}, and *bla*_{OXA-48}), has been evaluated using spiked rectal swabs (chapter 6). This evaluation demonstrated excellent performance of the assay, since an analytical sensitivity and specificity of 100% and limits of detection (LoD) comparable to a selective medium (ChromID Carba) were obtained. Moreover, when OXA-48 producing isolates with low MICs for meropenem are tested, the LoD of the Check-Direct CPE assay turned out to be superior over the LoD of the selective medium.

Regardless of the assay used, the possibility to detect carbapenemase genes directly from rectal swabs is an important feature for the surveillance of CPE, since appropriate infection control measures can be rapidly installed as a consequence. All molecular assays that can be used to test directly on DNA isolates from rectal swabs, have shown high sensitivities and are able to correctly identify the carbapenemase gene present in the rectal swab. But, when these molecular assays are compared to the reference method, which most often is culture using selective media, false-positive results are obtained resulting in specificities ranging from 89.8% - 99.4%.^{30,33–35} However, it is questionable whether the results of the real-time PCR assay really are false-positive. Vasoo *et al.* performed an accuracy study in which 46 previously characterized culture positive (all *bla*_{KPC}) swabs were included. While direct PCR was able to detect all samples correctly, the HardyCHROM ESBL agar and the CDC method only detected 35 and 36 samples respectively.³⁶ Similar results were described in a study testing 400 fecal samples. Here, the Check-Direct CPE assay was used and a carbapenemase gene was detected in two clinical samples (NDM/VIM, Ct-value 23.1; OXA-48, Ct-value 37.7), while no growth was seen using direct cultivation on ChromID Carba and ChromID OXA-48. Eventually, after overnight broth enrichment and subsequent culture, the carbapenemase producing isolate was detected in the sample being NDM/VIM positive in the Check-Direct CPE assay only.³⁷

Still, the reference method for CPE is culture. The question is however, should consequential infection control measures not be based on the results of molecular methods? The examples above illustrate the enhanced sensitivity of molecular testing over culture-based methods, probably due to a low concentration of CPE in the sample. But, so far, there is no data on the minimal concentration of CPE that has to be present for possible transmission of the CPE or its genes. Moreover, because of for instance environmental changes or antibiotic pressure, the composition of the gut flora changes continuously and the concentration of CPE can rapidly change from low to high. Therefore, when well-validated molecular testing identifies carbapenemase genes, that cannot be confirmed using culture based methods, samples should be attended positive. This is especially important in crucial settings such as for instance intensive care units.

Disadvantages of molecular testing

Although the molecular assays have clearly shown to have an added value for the identification of HRMOs, the use of molecular assays has some disadvantages. The main disadvantage is the fact that molecular methods are only able to detect the targeted genes. Therefore, continuously knowledge on the (regionally) most prevalent variants of HRMOs is essential to maintain the most optimal surveillance consisting of highly reliable molecular methods. As an example, the molecular assays for the detection of ESBL and CPE as described in this thesis are not suitable in Turkey and Brazil because of nationwide spread of respectively PER-1 ESBL^{38,39} and SPM carbapenemase^{40,41}.

In order to gain the knowledge needed, well developed studies should be performed periodically to determine the presence and distribution of all kinds of resistance genes, as is done with ESBLs by Voets *et al.*¹³ Another option is ongoing surveillance managed by a (reference) centre. Currently, the Dutch national reference laboratory RIVM manages surveillance of MRSA and determines the spa-type of each first isolate that is identified in a patient. The RIVM also manages the surveillance of isolates suspected to be a carbapenemase producer. In this surveillance, detection is performed using molecular detection of the carbapenemase genes *bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM} and *bla*_{OXA-48}, together with an in-house developed phenotypic method, the carbapenem inactivation method (CIM), that is based on the ability of the suspected isolate to inactivate meropenem within a disk.⁴² However, when a CPE suspected isolate shows carbapenem hydrolysis with the CIM test, but none of the five carbapenemase genes mentioned above is detected using molecular methods, no additional testing is performed. Therefore, the current surveillance on CPE is ineffective and no data can be generated on the presence of other genes encoding carbapenemase.

Another disadvantage of molecular testing is the costs per sample, which is considerably higher as compared to phenotypic methods. Obviously, this holds also true for the approaches as described in this thesis. However, in an (MRSA) outbreak situation molecular assays have shown to be cost-effective since the use of a rapid high throughput molecular screening reduces the number of screening tests and isolation days.⁴³ Also in case of ESBL, molecular testing has shown to be cost-effective.¹⁵ Comparing results and costs of the ESBL E-test with the costs of a DNA micro-array, used for the confirmation of ESBL in hospitalized patients, revealed the cost effectiveness of the DNA micro-array. This is associated with the prevention of unnecessary isolation days because of the lower specificity of the E-test. It must be stressed though, that this study only included the costs of the reagents of both confirmation methods and did not take the hands-on time into account, which is very high for the DNA micro-array. Nevertheless, when such system could be automated or replaced by a less laborious molecular method, the high specificity of molecular methods would be an important feature in obtaining cost effectiveness compared to phenotypic methods.

Conclusions and future perspectives

As a result of the increasing use of antibiotics, combined with the lack of development of new drugs, antimicrobial resistance has been emerging worldwide and now is a global problem. To prevent further spread of resistance, surveillance programs have been developed in order to rapidly identify highly resistant microorganisms. For this purpose, we introduced different molecular methods for the detection of the most prevalent and clinically important genes encoding MRSA, ESBL and CPE that showed to be important to reduce time-to-results. Moreover, the results presented in this thesis clearly show improved identification of HRMOs since high sensitivity and specificity of the molecular assays is obtained.

To further improve the turn-around-time for the detection of multi-resistant bacteria, more and more molecular POCT assays will become available. In these, companies such as Cepheid and Becton Dickinson (BD) have developed systems with supplementary assays for POCT that can obtain results within 1-2 hours and only need a few minutes hands on time. Next to the already proven successful MRSA assays^{44,45}, POCT is also introduced for detection of genes encoding CPE. Cepheid was the first to introduce a POCT assay for the detection of *bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM} genes using the GeneXpert.³⁵ Recently, they introduced a new assay that is also able to detect *bla*_{OXA-48} and *bla*_{IMP}.⁴⁶ Check-Points and BD cooperated in the development of another POCT assay for the detection of carbapenemase genes. The Check-Direct CPE assay that has been evaluated as part of this thesis was adapted and applied on the BD Max system which is now available for POCT. In the same way, Check-Points and BD will also launch a POCT assay that is able to detect ESBL genes using the BD Max system. The added value of POCT for ESBL and CPE producers in a clinical setting has not yet been evaluated. Comparing these assays to the methods as recommended by the NVMM guideline, as is done with MRSA¹⁹, should be performed to gain important information on the added value of these POCT assays.

In the next coming years, novel molecular methods such as next-generation sequencing (NGS) will possibly be introduced in routine settings for the detection of a wide variety of resistance genes within one assay. Pathogenica has developed the HAI BioDetection system, an assay based on NGS using approximately 300 probes.⁴⁷ This assay is able to detect the presence of 12 different pathogens and a wide range of resistance genes. Moreover, the kit can also be used to type the strains and provide a phylogenetic tree for sets of isolates, which can be very useful in outbreak situations. Although not yet evaluated, this NGS assay has been developed to be used directly on clinical samples such as swabs, blood culture or urine.

This thesis focuses on the molecular detection of genes encoding MRSA, ESBL and CPE. Next to these well-known resistance markers, dissemination of other important resistance mechanisms is also observed. Resistance to for instance ciprofloxacin (i.e. quinolone) and vancomycin (i.e. glycopeptide) is increasingly reported and the latter has been involved in hospital outbreaks worldwide. Since resistance to ciprofloxacin and vancomycin is also mediated by transferrable genes (*qnrA* and *vanA/vanB* respectively)^{48,49}, rapid detection of these genes in addition to the

detection of MRSA, ESBL and CPE seems to be a logical next step. So far, several real-time PCRs, including POCT for the detection of *vanA/vanB*, have already been developed.^{50–53} When the current guidelines have to be revised (2017), molecular detection of not only MRSA, ESBL and CPE, but also of the genes mediating resistance to ciprofloxacin and vancomycin should be included.

With the increasing introduction of a wide range of different molecular applications, detection of HRMOs using molecular methods will also be performed more and more. Since these assays have proven to reduce the time-to-result, infection control practitioners are subsequently able to prevent spread of the detected HRMO by introducing adequate infection control measures. To achieve the biggest effect, it might become obligatory that all hospitals introduce rapid molecular diagnostics for the detection of HRMOs. Although not all laboratories have the facilities to perform molecular diagnostics, introduction of POCT or cooperation with other laboratories that do have these facilities makes it possible to further establish rapid identification methods.

When all laboratories have implemented rapid, reliable molecular assays and subsequent infection control measures can be installed when necessary, an important step is achieved to slow down the emergence of antimicrobial resistant strains.

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ABBREVIATIONS

ARI-1:	<i>Acinetobacter</i> resistant to imipenem
BCII:	<i>Bacillus cereus</i> II
BEL:	Belgium ESBLs
BES:	Brazilian ESBLs
CA-MRSA:	community-associated MRSA
Ccr:	cassette chromosome recombinases
CDC:	center for disease control and prevention
CFU:	colony forming units
CPE:	carbapenemase producing <i>Enterobacteriaceae</i>
CphA:	carbapenemase hydrolysing <i>Aeromonas</i>
Ct:	Cycle threshold
CTX-M:	cefotaximase - Munich
DIM:	Dutch imipenemase
DNA:	desoxyribonucleic acid
EARS-net:	European Antimicrobial Resistance Surveillance Network
ECDC:	European center for disease prevention and control
ESBL:	extended-spectrum beta-lactamase
FEC:	Fujisawa <i>E. coli</i>
GES:	Guyana ESBLs
GIM:	German imipenemase
HA-MRSA:	hospital-associated MRSA
HRMO:	highly resistant microorganisms
IC:	internal inhibition control
ICU:	intensive care unit
IMI:	imipenemase
IMP:	active on imipenem
IPM:	imipenem
KPC:	<i>Klebsiella pneumoniae</i> carbapenemase
L1:	characteristic of <i>Stenotrophomonas maltophilia</i>
LA-MRSA:	livestock-associated MRSA
LM-PCR:	ligation-mediated real-time PCR
LoD:	limit of detection
MALDI-TOF:	matrix-assisted laser desorption ionization–time of flight
MBL:	metallo-beta-lactamase
MIC:	minimal inhibitory concentration
MEM:	meropenem
MHT:	modified hodge test
MLST:	multi-locus sequence typing
MRCNS:	methicillin resistant coagulase negative staphylococci

MRSA:	methicillin resistant <i>Staphylococcus aureus</i>
MSSA:	methicillin susceptible <i>Staphylococcus aureus</i>
NDM:	New-Delhi metallo-beta-lactamase
NGS:	next-generation sequencing
nM:	nano-molar
NMC-A:	not metallo-enzyme carbapenemase A
NVMM:	Nederlandse vereniging voor medische microbiologie
orfX:	open reading frame X
OXA:	hydrolysis of oxacillin
PBP:	penicillin binding protein
PCR:	polymerase chain reaction
PER:	<i>Pseudomonas</i> extended resistance
PhHV:	Phocine herpes virus
PMB:	phenol mannitol broth
POCT:	point of care testing
PVL:	panton-valentine leukocidin
RIVM:	Dutch National Institute for Public Health and the Environment
rpm:	rotation per minute
SCCmec:	Staphylococcal cassette chromosome <i>mec</i>
S&D:	search and destroy
SFO:	<i>Serratia fonticola</i>
SHV:	sulphydryl variable
SIM:	Seoul imipenemase
SME:	<i>Serratia marcescens</i> enzyme
SPM:	Sao Paulo metallo-beta-lactamase
ST:	sequence type
TAT:	turnaround time
TEM:	Temoneira (patient's name)
TLA:	Tlahuicas (Indian tribe)
Toho:	Toho university school of medicine omori hospital
TSB:	tryptone soya broth
VEB:	Vietnam extended-spectrum beta-lactamase (ESBLs)
VIM:	Verona imipenemase
WIP:	working party for infection control
WHO:	world health organization

SUMMARY

Antimicrobial resistance is emerging worldwide and considered one of the major threats in healthcare. This increasing resistance has major consequences, not only for the correct treatment of the individual patient, but also for hospitals where it is continuously challenging infection control and hygiene procedures. Finally, worldwide dissemination of antimicrobial resistance is seen and can now rapidly emerge everywhere in the world. Although guidelines have been developed in order to identify carriers of highly resistant microorganisms (HRMOs) as rapid as possible, the recommended method of laboratory detection of HRMOs is mostly time-consuming. Moreover, since the laboratory detection mostly consists of phenotypic methods, performance of these methods can be insufficient. The aim of this thesis was to improve laboratory detection of HRMOs by introducing molecular assays able to detect all kind of resistance genes, and show the clinical importance of these molecular assays for the rapid and reliable detection of resistance genes. **Chapter 1** gives an overview on the mechanisms of resistance, epidemiology and current methods to detect the most prevalent and clinically important HRMOs; methicillin resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase (ESBL) and carbapenemase producing *Enterobacteriaceae* (CPE). **Chapter 2** describes the evaluation of a ligation-mediated real-time PCR assay that is able to detect the most prevalent genes mediating ESBL. The performance of the molecular assay was compared with the performance of the combination disc test (CDT), a phenotypic method that is recommended to be used for the confirmation of ESBLs by the NVMM guideline on the laboratory detection of HRMOs. Therefore, 172 prospective putative ESBL-positive *Enterobacteriaceae* isolates from clinical specimens based on Vitek2 results were included in this study. Positive ESBL results were obtained in 100 and 95 isolates using CDT and the real-time PCR assay, respectively. Seven discrepancies were identified, of which six isolates were detected CDT positive, real-time PCR negative. The remaining discrepant isolate was detected CDT negative, real-time PCR positive. Discrepancy testing included both the Check-MDR CT103 microarray and PCR and sequencing and confirmed the results obtained using the real-time PCR assay. It was concluded that the performance of the ligation-mediated real-time PCR assay was superior over the recommended phenotypic method. Moreover, the molecular assay provides an important reduction in turnaround time (~4.5 h versus overnight incubation using CDT) for ESBL confirmation. **Chapter 3** focuses on the distribution of ESBL genes in our region. Using the Check-ESBL assay, a microarray based system able to detect TEM, SHV or CTX-M genes and at the same time specifies the CTX-M group, we were able to generate an overview on the ESBL genes present in our region. In 247 isolates, a total of 274 ESBL genes were identified, of which CTX-M1 group was detected predominantly, followed by CTX-M9 group, SHV, TEM and CTX-M2 group. Furthermore, when four different patient categories were distinguished, we were able to determine differences in the identified ESBL genes per patient category. Here, it was shown that CTX-M9 group ESBLs were significantly more prevalent in ICU patients, whereas SHV ESBLs were more common in hospitalized patients than in outpatients. **Chapter 4** presents the results of our MRSA screening approach over a 2-year period. This MRSA screening approach consists of overnight selective broth enrichment, followed by real-time PCR assays targeting *mecA*, *mecC*

and the *S. aureus*-specific SA442-gene, with subsequent confirmation using a staphylococcal cassette chromosome *mec* element (SCC*mec*)-*orfX*-based real-time PCR assay (GeneOhm MRSA assay) and culture. The performance of the real-time PCR assays was evaluated using a set of 104 assorted MRSA isolates and showed high sensitivity and specificity. During the 2-year study-period, 13,387 samples were analyzed for the presence of MRSA. Based on the results of the real-time PCR assays only, 95.2% of the samples could be reported as negative within 24 h after arrival at the laboratory. Eventually, after detection performed using the molecular assays and subsequent culture, 2.6% of all samples was reported as MRSA positive. This molecular screening approach proved to be an accurate method for obtaining reliable negative results within 24 h after arrival at the laboratory and contributes to improvement of infection control programs, especially in areas with a low MRSA prevalence. **Chapter 5** describes the clinical application of a likewise approach for the detection of CPE. Using two different broths, followed by detection using a commercially available ligation-mediated real-time PCR assay (Check-MDR Carba assay), we were able to determine the presence of the most prevalent carbapenemase genes (KPC, VIM, NDM, IMP, OXA-48) in rectal swab samples. Over a 14-month period, 454 individual patients were screened using this method. In samples obtained from 6 patients (1.3%), a CPE could be detected using the real-time PCR assay, all of which could be confirmed using culture. Of these six patients, that all had been hospitalized in countries known for a high prevalence of CPE producers, four patients carried an OXA-48 producer, while in the two remaining patients a KPC-producer was identified. Since early identification and isolation of carriers are key components of an effective infection control strategy in hospitals, this method may be a major improvement in the challenge to reduce the spread of CPE. **Chapter 6** describes the evaluation of a new real-time PCR assay (Check-Direct CPE assay) that has been developed for the detection of carbapenemase genes KPC, VIM, NDM, OXA-48 directly from DNA isolated from rectal swabs. Using 83 non-duplicate isolates, the assay demonstrated 100% sensitivity and sensitivity. To determine the performance of the assay directly from DNA isolated from rectal swabs, we spiked rectal swabs with serial dilutions of 19 different carbapenemase producers. Limits of detection of the real-time PCR assay was determined and compared with a selective medium (ChromID Carba), that is recommended by the NVMM guideline to screen for the presence of CPE. Besides OXA-48 producers with low MICs for carbapenem antibiotics, that are poorly detected by this selective medium, the limits of detection of the real-time PCR assay were comparable to the limits of detection obtained with the ChromID Carba. Results can be obtained within 3h using the Check-Direct CPE assay, in comparison with overnight incubation followed by subsequent confirmation using selective media. Therefore, we concluded that the real-time PCR assay looks promising as a CPE screening method and can be an important improvement in infection control programs. In the final **chapter 7**, the results of the preceding chapters are discussed and future perspectives on molecular detection of antimicrobial resistance are given.

SAMENVATTING

Wereldwijd is er een toename te zien in antimicrobiële resistentie, welke wordt gezien als één van de grootste dreigingen van de gezondheidszorg. Deze toenemende resistentie heeft grote gevolgen, niet alleen voor de correcte therapie voor de individuele patiënt, maar ook voor ziekenhuizen waar resistentie een uitdaging is voor hygiëne en infectiepreventie maatregelen. Tenslotte is wereldwijde verspreiding van antimicrobiële resistentie nu al een feit, en is het reëel om te veronderstellen dat de resistentie overal ter wereld verder toe gaat nemen. Om dragers van bijzonder resistente micro-organismen (BRMOs) zo snel mogelijk te kunnen identificeren, zijn er specifieke richtlijnen opgesteld. Zo is er een richtlijn welke aanbevelingen doet omtrent laboratorium diagnostiek voor het aantonen van de BRMOs. De hierin aanbevolen detectiemethoden zijn voornamelijk phenotypische methoden, welke niet alleen een lange turn-around-time hebben, maar ook niet in staat zijn om alles correct te detecteren. Het doel van het onderzoek beschreven in dit proefschrift is om de detectie van BRMOs te verbeteren, waarbij moleculaire technieken belangrijk zijn voor snelle en betrouwbare detectie van resistentiegenen. **Hoofdstuk 1** geeft een overzicht van de resistentiemechanismen, epidemiologie en huidige detectie methoden van de meest voorkomende en klinisch relevante BRMOs; meticilline resistente *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase (ESBL) en carbapenemase producerende Enterobacteriaceae (CPE). **Hoofdstuk 2** beschrijft vervolgens de evaluatie van een ligatie-afhankelijke real-time PCR assay, welke de meest voorkomende ESBL genen (TEM, SHV en CTX-M) kan detecteren. Voor deze evaluatie zijn de resultaten van de moleculaire assay vergeleken met de resultaten van de combination disc test (CDT), een phenotypische methode welke door de NVMM richtlijn (laboratorium diagnostiek van BRMOs) wordt aanbevolen voor de confirmatie van ESBLs. Voor deze vergelijking zijn 172 klinische Enterobacteriaceae isolaten getest, welke op basis van Vitek2 resultaten verdacht waren voor ESBL. Honderd isolaten waren ESBL positief volgens de CDT en 95 ESBL positief volgens de real-time PCR assay. Er werden zeven discrepante resultaten waargenomen, waarvan zes isolaten positief waren met de CDT, maar negatief met de real-time PCR assay. Het resterende isolaat was CDT negatief, terwijl het positief was met de real-time PCR assay. Discrepantie analyse werd uitgevoerd met de Check-MDR CT103 micro-array en PCR met sequencing, waarbij de resultaten van de real-time PCR allemaal werden bevestigd. Naar aanleiding van dit onderzoek was de conclusie dat de werking van de ligatie-afhankelijke real-time PCR assay superieur is ten opzichte van de aanbevolen phenotypische methode. Daarnaast zorgt het gebruik van de moleculaire methode ook voor een belangrijke reductie in de turn-around-time (~4,5 uur versus overnacht incubatie bij de CDT) voor ESBL confirmatie. In **hoofdstuk 3** ligt de focus op de distributie van ESBL genen in onze regio. In deze studie wordt gebruik gemaakt van de Check-ESBL assay, een micro-array welke niet alleen TEM, SHV en CTX-M genen kan identificeren, maar tegelijkertijd ook de CTX-M-groep kan specificeren. Met behulp van deze micro-array werd een overzicht verkregen van de ESBL genen die in de regio voorkomen. In de 247 geteste isolaten zijn in totaal 274 ESBL genen aangetoond, waarbij CTX-M1 het vaakst werd geïdentificeerd, gevolgd door CTX-M9, SHV, TEM en CTX-M2. Tevens zijn de in de studie

geïncubeerde patiënten onderverdeeld in vier verschillende categorieën, zodat mogelijke verschillen in het voorkomen van ESBL genen konden worden aangetoond. Hieruit bleek dat de CTX-M9 ESBL significant meer werd gevonden in IC-patiënten, dan in alle andere patiënten. Daarnaast werd duidelijk dat SHV ESBL over het algemeen meer te vinden is bij patiënten welke opgenomen zijn in het ziekenhuis, dan bij patiënten welke niet in het ziekenhuis liggen.

Hoofdstuk 4 beschrijft de resultaten van een moleculaire screeningsmethode voor de detectie van MRSA, gedurende een periode van 2 jaar. De methode bestaat uit een overnacht verrijking in een bouillon, waarna real-time PCR assays worden uitgevoerd gericht op het *mecA*, *mecC* en het *S. aureus*-specifieke SA442-gen. Deze initiële stap wordt gevolgd door confirmatie met een andere real-time PCR assay, de GeneOhm MRSA assay, welke het Staphylococcal cassette chromosome *mec* element (SCC*mec*) gecombineerd met het *orfX*-gen detecteert. Bij een positief resultaat van beide assays vindt er uiteindelijk kweek plaats. De prestatie van de real-time PCR assays werd geëvalueerd met 104 verschillende MRSA stammen, met als resultaat een hoge sensitiviteit en specificiteit. Gedurende de 2-jaar durende studieperiode zijn in totaal 13.387 monsters gescreend op de aanwezigheid van MRSA. Op basis van alleen de real-time PCR assays, kon 95.2% van de monsters als negatief worden uitgeslagen binnen 24 uur na binnenkomst op het laboratorium. Uiteindelijk kon in 2.6% van alle monsters een MRSA gekweekt worden. Deze moleculaire screeningsmethode heeft bewezen een accurate methode te zijn, welke in staat is om binnen 24 uur na binnenkomst op het laboratorium betrouwbare resultaten te genereren. Deze snelle diagnostische methode draagt bij aan verbetering van infectie preventie programma's, zeker wanneer het een populatie betreft waar MRSA weinig voorkomt. In **hoofdstuk 5** wordt het gebruik van een vergelijkbare methode beschreven voor de detectie van CPE. Hierbij worden twee verschillende bouillons gebruikt, waarbij bij rectum uitstrijken na ophoping detectie van de meest voorkomende CPE genen (KPC, VIM, NDM, IMP, OXA-48) plaatsvindt, gebruikmakend van een commerciële ligatie-afhankelijke real-time PCR assay (Check-MDR Carba assay). Gedurende een periode van 14 maanden, zijn rectum uitstrijken van 454 verschillende patiënten gescreend met behulp van deze methode. In rectum uitstrijken afkomstig van zes patiënten (1.3%) kon een CPE worden gedetecteerd met behulp van de real-time PCR assay. Na afenting van deze bouillons werd in alle gevallen een CPE gekweekt, waarmee de resultaten van de real-time PCR assay werden geconfirmeerd. De zes patiënten waren allemaal opgenomen in ziekenhuizen in landen waarvan bekend is dat er een hoge prevalentie van CPE is. Vier patiënten bleken drager te zijn van een OXA-48 producerend micro-organisme en bij de overige twee patiënten kon een KPC worden geïdentificeerd. Aangezien een zo vroeg mogelijke detectie, met bijbehorende isolatie maatregelen, een uitzonderlijk belangrijk onderdeel is van een effectief infectie preventie beleid, zal deze methode mogelijk een enorme bijdrage kunnen leveren aan het voorkomen van verspreiding van CPE. **Hoofdstuk 6** van dit proefschrift beschrijft de evaluatie van een nieuwe real-time PCR assay (Check-Direct CPE assay), ontwikkeld voor de detectie van de carbapenemase genen KPC, VIM, NDM en OXA-48, direct in DNA geïsoleerd uit rectum uitstrijken. Evaluatie van deze assay met 83 verschillende

stammen liet een sensitiviteit en specificiteit van 100% zien. Om de betrouwbaarheid van de assay aan te tonen voor de detectie van CPE genen in DNA geïsoleerd uit klinisch materiaal, is gebruik gemaakt van rectum uitstrijken gespiked met verdunningsreeksen van 19 verschillende carbapenemase producers. Hiermee is de detectielimiet van de real-time PCR assay bepaald en vergeleken met de detectielimiet van een door de NVMM richtlijn aanbevolen CPE screenings medium (ChromID Carba). Behalve OXA-48 producerende isolaten met lage MICs voor carbapenem antibiotica, welke niet of nauwelijks werden gedetecteerd door de ChromID carba, was de detectielimiet van beide methoden vergelijkbaar. Detectie met de Check-Direct CPE assay genereert resultaten binnen 3 uur, dit in tegenstelling tot selectieve media waarbij minstens overnacht incubatie nodig is, gevolgd door een additionele confirmatie stap in geval van CPE verdachte kolonies. Als resultaat van deze studie kon worden geconcludeerd dat de real-time PCR assay een veelbelovende assay is, welke als screeningsmethode mogelijk een belangrijk onderdeel kan zijn van infectiepreventie programma's. In het laatste hoofdstuk van dit proefschrift, **hoofdstuk 7**, worden de resultaten van de vorige hoofdstukken bediscussieerd en wordt vooruitgeblikt op moleculaire detectie van antimicrobiële resistentie in de toekomst.

VALORIZATION PARAGRAPH

Worldwide, an emerging increase in antimicrobial resistance is observed. This is alarming since antimicrobial resistance might not only be responsible for increased morbidity and mortality for the individual patient, but has also been responsible for major hospital outbreaks.

The aim of this research was to study different strategies and methods to improve the laboratory detection of highly resistant micro-organisms (HRMOs) and at the same time decrease the time to result. The rapid and correct detection of HRMOs is of utmost importance for the individual patient, in order to obtain the adequate treatment as rapid as possible. Moreover, the improved laboratory detection of HRMOs is important for the implementation of the correct infection prevention measures within hospitals.

The results of the research in chapter 2 showed that phenotypical detection of extended-spectrum beta-lactamases (ESBLs) can produce false positive results, compared to the molecular detection of ESBL. Since the current Dutch guideline recommends to nurse ESBL positive patients in isolation, these false positive results can consequently be responsible for installing incorrect isolation procedures. Next to the additional costs associated with isolated nursing, it is also very inconvenient and stressful for both patient and nursing personnel. Therefore, the use of molecular detection to confirm ESBL suspected isolates is important to prevent unnecessary costs and frustration associated with incorrect isolation procedures.

All studies presented in this thesis, describe a molecular method that is responsible for a reduction in time to result, when compared to conventional phenotypical methods. This reduction in time to result is important to prevent further spread of HRMOs within the hospital, since corresponding infection prevention measures can be installed earlier. Moreover, when an accidental HRMO is identified from a sample obtained from a hospitalized patient, molecular methods as described in this thesis can be used in order to rapidly generate an overview on the possible spread of the corresponding HRMO. Both the early prevention of further spread of multi-resistant bacteria and the possibility to avoid unnecessary isolation days (because of the rapid detection of HRMOs) will save hospitals a high amount of money. Consequently, this saved money can be used elsewhere in the hospital for further improvement of healthcare.

Eventually, when molecular methods as described in this thesis are widely introduced, the rapid and correct detection of HRMOs and the consequences as described above will be responsible for reducing worldwide spread of HRMOs, which is one of the focus points of the World Health Organization (WHO). This reduction in worldwide spread of HRMOs hopefully gives time for the development of new antimicrobial agents to treat the current HRMOs.

The results reported in this thesis are of considerable interest to clinical microbiologists, molecular biologists and infection prevention workers. Moreover, the results can also be helpful to policymakers. The current Dutch guideline for the laboratory detection of HRMOs, as developed by a working group of the Dutch society for medical microbiology (NVMM), describes recommendations that are mainly based on conventional phenotypical methods. With the results of the research as presented in this thesis, new insights on the genotypic detection of HRMOs are obtained. These insights can be used when the current guideline for the laboratory detection of HRMOs is revised.

In the past decade, a number of different techniques, developed by different researchers or companies, have been introduced for the laboratory detection of HRMOs. The results of the research as described in this thesis will provide a solid basis for near future molecular diagnostic assays as well as for improvement of currently available methods. In chapter 3, we describe the distribution of ESBL genes found in the east of the Netherlands. The results of this study, combined with other studies, will be important to companies that develop molecular assays for the rapid detection of ESBL genes. The scientific evidence described in this thesis and other studies will enable companies to make decisions on the relevant genes that have to be included into a new assay. The same holds true for the results of chapters 4 and 5, in which respectively the different types of MRSA and CPE are described that have been identified with our routine screening method.

During the studies performed for this thesis, a wide variety of clinical isolates have been analyzed. These clinical isolates are stored and included in the corresponding collection and database of the Laboratory for Medical Microbiology and Medical Immunology. Since the presence and type of resistance-genes in these clinical isolates is well known, this collection can be very valuable for future studies and validation of (commercial) molecular diagnostic assays. The results of such validations can both be used for publications in peer reviewed journals, as well as for CE marking of products.

To conclude, the prevalence of antibiotic resistance is increasing, resulting in a worldwide spread of these highly resistant micro-organisms. This thesis described evaluations and implementations of molecular methods for the correct and rapid detection of HRMOs. These methods can therefore contribute to the prevention of further spread of HRMOs, resulting in time needed to develop new antimicrobial agents to be used for the treatment of infections caused by the HRMOs.

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Dank je wel!

Roel

CURRICULUM VITAE AND LIST OF PUBLICATIONS

Curriculum vitae

Roel Hermanus Theodorus Nijhuis werd geboren op 7 maart 1983 te Lichtenvoorde. Na het behalen van het HAVO diploma aan scholengemeenschap Marianum te Groenlo, begon hij in 2000 aan de HBO opleiding biologie en medisch laboratorium onderzoek aan de Saxion Hogeschool Enschede. Deze opleiding heeft hij in 2004 afgerond, waarmee hij zichzelf Bachelor of applied science mocht noemen. Direct na het behalen van zijn diploma is hij gestart als allround microbiologisch analist op het laboratorium voor medische microbiologie en infectieziekten van de Isala klinieken te Zwolle. In mei 2006 is hij vervolgens gestart als allround microbiologisch analist op het laboratorium voor medische microbiologie en medische immunologie van het Rijnstate ziekenhuis Arnhem, waar hij per 1 maart 2008 werd aangesteld als aandachtsanalist moleculair. In 2010 startte hij met het promotie onderzoek aan die zelfde afdeling. Dit onderzoek werd uitgevoerd onder supervisie van prof. dr. P.H.M. Savelkoul (eerst VUMC Amsterdam, later UMC Maastricht) en dr. A.A. van Zwet (Rijnstate, Arnhem). In januari 2015 is hij, onder supervisie van dr. E.C. Claas, gestart met de 2-jarige opleiding tot medisch moleculair microbioloog (MMM-er) aan het Leids Universitair Medisch Centrum.

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