NVMM Guideline

Laboratory detection of highly resistant microorganisms (HRMO)

Authors

Dr. A.T. Bernards, clinical microbiologist

Prof. dr. M.J.M. Bonten, clinical microbiologist

Dr. J. Cohen Stuart, clinical microbiologist

Dr. B.M.W. Diederen, clinical microbiologist

Dr. W.H.F. Goessens, medical microbiologist

Prof. dr. H. Grundmann, clinical microbiologist

Prof. dr. J.A.J.W. Kluytmans, clinical microbiologist

Drs. M.F.Q. Kluytmans - van den Bergh, epidemiologist

Dr. M.A. Leverstein - van Hall, clinical microbiologist

Prof. dr. J.W. Mouton, clinical microbiologist

Dr. N. al Naiemi, clinical microbiologist

Dr. A. Troelstra, clinical microbiologist

Prof. dr. C.M.J.E. Vandenbroucke - Grauls, clinical microbiologist

Prof. dr. M.C. Vos, clinical microbiologist

Prof. dr. A. Voss, clinical microbiologist

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Colofon

PO box 21020

8900 JA Leeuwarden

The Netherlands

T: (+31) 058-2939249

E: nvmm@knmg.nl

W: http://www.nvmm.nl

Information about the guideline: bureau@nvmm.nl

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TABLE OF CONTENTS

CHAPTER 1 - GENERAL INTRODUCTION

1.1 Background

Antimicrobial resistance is rapidly emerging worldwide, and affects both healthcare and community settings, and intensive livestock agriculture [EARSS 2008, SWAB 2010, VANTURES 2008]. The increase in antimicrobial resistance concerns not only the number of individuals infected or colonised with antimicrobial resistant microorganisms, but also the diversity of underlying resistance mechanisms [Paterson 2005, Queenan 2007]. Antimicrobial resistance hampers the options for antimicrobial therapy, and is associated with increased morbidity, mortality and healthcare costs [Carmeli 2002, Cosgrove 2005, Mauldin 2010].

National guidelines for the control of highly resistant microorganisms (HRMO) have been developed by the Dutch Workingparty on Infection Prevention (WIP). [Kluytmans 2005, WIP 2005, WIP 2007] HRMO are defined as microorganisms that 1) are known to cause disease; 2) have acquired an antimicrobial resistance pattern that hampers (empirical) therapy, and 3) have the potential to spread in healthcare facilities if – in addition to standard precautions – no transmission-based precautions are taken.

The efficient control of HRMO strongly depends on the adequate laboratory detection of antimicrobial resistance [Metan 2005]. The implementation of rapid and accurate laboratory detection methods may improve the timely initiation of appropriate antimicrobial therapy as well as infection control measures to prevent the spread of antimicrobial resistant microorganisms within healthcare facilities [Creamer 2010, Galar 2012]. Furthermore, it may decrease the duration of pre-emptive isolation precautions [Wassenberg 2010], and prevent the inappropriate institution of contact tracing.

1.2 Objective

This guideline provides recommendations on the appropriate use of currently available diagnostic laboratory methods for the timely and accurate detection of HRMO, as defined by the WIP [Kluytmans 2005, WIP 2005], in patients and healthcare workers. Herewith, it aims to standardise and improve the diagnostic laboratory procedures that are used for the detection of HRMO in Dutch medical microbiology laboratories.

1.3 Target group

This guideline is aimed at clinical microbiologists, infection control practitioners, laboratory technicians and medical microbiology laboratories that are responsible for the detection of HRMO in patients and healthcare workers in the Netherlands.

1.4 Realisation

The development of this guideline was initiated by the Netherlands Society for Medical Microbiology (NVMM) in 2009, and funded by the Stichting Kwaliteitsgelden Medisch Specialisten (SKMS).

1.5 Working group

This guideline has been developed by a working group that was instituted by the NVMM in 2010. The working group consists of clinical microbiologists, and medical microbiologists with known expertise in the field of the laboratory detection of antimicrobial resistance. The working group members represent both university and non-university centres from different regions of the Netherlands. The working group members are jointly responsible for the full text of this guideline.

1.5.1 Working group members

The following persons participated in the development of the guideline:

- Dr. A.T. Bernards, clinical microbiologist (LUMC, Leiden)
- Prof. dr. M.J.M. Bonten, clinical microbiologist (UMC Utrecht, Utrecht)
- Dr. J. Cohen Stuart, clinical microbiologist (UMC Utrecht, Utrecht)
- Dr. B.M.W. Diederen, clinical microbiologist (Regional Laboratory of Public Health Haarlem, Haarlem)
- Dr. W.H.F. Goessens, medical microbiologist (Erasmus MC, Rotterdam)
- Prof. dr. H. Grundmann, clinical microbiologist (UMC Groningen, Groningen)
- Prof. dr. J.A.J.W. Kluytmans, clinical microbiologist (Amphia Hospital, Breda / St. Elisabeth Hospital, Tilburg / TweeSteden Hospital, Tilburg / VUmc, Amsterdam)[1]
- Drs. M.F.Q. Kluytmans - van den Bergh, epidemiologist (Amphia Academy Infectious Disease Foundation, Breda)[2]
- Dr. M.A. Leverstein- van Hall, clinical microbiologist (Bronovo, Den Haag / RIVM, Bilthoven)
- Prof. dr. J.W. Mouton, clinical microbiologist (UMC St. Radboud, Nijmegen)
- Dr. N. al Naiemi, clinical microbiologist (Regional Public Health Laboratory, Enschede)
- Dr. A. Troelstra, clinical microbiologist (UMC Utrecht, Utrecht)
- Prof. dr. C.M.J.E. Vandenbroucke–Grauls, clinical microbiologist (VUmc, Amsterdam)
- Prof. dr. M.C. Vos, clinical microbiologist (Erasmus MC, Rotterdam)[1]
- Prof. dr. A. Voss, clinical microbiologist (Canisius-Wilhelmina Hospital, Nijmegen / UMC St. Radboud, Nijmegen)

1 Chairman

2 Secretary

1.5.2 Conflict of interest

No potential conflict of interest disclosed

Dr. A. T. Bernards

Prof. dr. M.J.M. Bonten

Dr. B.M.W. Diederen

Dr. W.H.F. Goessens

Prof. dr. H. Grundmann

Drs. M.F.Q. Kluytmans – van den Bergh

Prof. dr. J.W. Mouton

Dr. N. al Naiemi
Dr. A. Troelstra
Prof. dr. M.C. Vos
Prof. dr. A. Voss

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Dr. J. Cohen Stuart: research (MSD, Pfizer); conference visit (Pfizer, Rosco)
Prof. dr. J.A.J.W. Kluytmans: research (AstraZeneca, Check-Points); consultation (bioMérieux, Pfizer)
Dr. M.A. Leverstein – van Hall: research (Oxoid, Rosco)
Prof. dr. C.J.M.E. Vandenbroucke – Grauls: conference visit (bioMérieux)

1.6 Topics

1.6.1 Highly resistant microorganisms (HRMO)

In accordance with the definitions of HRMO issued by the WIP [Kluytmans 2005, WIP 2005] this guideline provides recommendations on the following combinations of microorganism, susceptibility pattern and/or resistance mechanisms: 1) *Staphylococcus aureus*: methicillin resistance; 2) *Streptococcus pneumoniae*: penicillin(group) resistance and vancomycin resistance; 3) *Enterococcus faecium*: penicillin(group) resistance and vancomycin resistance; 4) Enterobacteriaceae: extended-spectrum beta-lactamases, carbapenemases, quinolone resistance, and aminoglycoside resistance; 5) non-fermenting gram-negative bacteria (*Acinetobacter* spp., *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*): ceftazidime- and piperacillin resistance (acquired beta-lactamases), quinolone resistance, aminoglycoside resistance, carbapenemases, and co-trimoxazole resistance.

1.6.2 Aspects of laboratory detection

This guideline provides recommendations on the laboratory detection of HRMO. For recommendations on measures to prevent transmission of HRMO the working group refers to the relevant guideline of the WIP [WIP 2005]. Several aspects of the laboratory detection of HRMO are covered in this guideline, i.e. 1) detection of carriage: culture sites, number of cultures, culture materials and transport, and timing of cultures in healthcare workers; 2) laboratory methods: direct molecular detection, solid agar media, broth enrichment, pooling of samples, identification and susceptibility testing (including screening, phenotypic and genotypic confirmation, and quality control); 3) contact tracing: adjusting diagnostic methods in case of a ‘known’ strain and molecular typing; 4) reporting: laboratory- and patient information system; and 5) quality indicators: laboratory turnaround time.

1.6.3 Surveillance of resistance

Although the surveillance of antimicrobial resistance is not part of this guideline on the laboratory detection of HRMO, the working group does recommend medical microbiology laboratories to participate in national surveillance programs that aim on the monitoring and early detection of trends in antimicrobial resistance on a national level.

1.6.4 Quality indicators

Quality indicators are tools that can be used to measure selected aspects of the quality of care. Laboratory medicine quality indicators should focus on those areas considered most likely to have important consequences on patient care and health outcomes. Also, quality indicators should be defined as objective measures that can be implemented in a consistent and comparable manner across settings and over time [Shahangian 2009]. Quality indicator data should be collected over time to identify and continuously monitor problems, and improve performance by identifying and implementing effective interventions [Shahangian 2009].

The quality of diagnostic laboratory methods is not only dependent on the accuracy of the test results, but also on the timely availability of these results. Therefore, laboratory turnaround time (LTAT) is often used as a key performance indicator of laboratory performance [Hawkins 2007, Howanitz 2001]. The timeliness of reporting the presence of antimicrobial resistant microorganisms may improve the timely initiation of appropriate antimicrobial therapy as well as appropriate infection control measures [Creamer 2010, Galar 2012, Wassenberg 2010]. Publications on LTAT as a quality indicator for medical microbiology laboratory are limited. Nevertheless, the working group has decided to include LTAT in this guideline as an internal quality indicator that is aimed at improving the quality of the individual medical microbiology laboratory practice. LTAT is defined as the time from arrival of the clinical specimen in the laboratory until the time of report [Breil 2011]. Given the limited available evidence, no quantitative target standards have been set at this time. Recommended qualitative target standards pertain to the documentation and monitoring of LTAT for both positive and negative laboratory results. Recommended measures to periodically monitor LTAT are the mean, median, and 90^{th} percentile [Valenstein 1989]. In addition, it is recommended to monitor and investigate outliers (e.g. $>90^{th}$ percentile) [Steindel 1999], in order to improve performance over time [Steindel 1997]. Finally, it is important to realise that improving timeliness should not be at the expense of the accuracy of test results.

1.7 Methods

1.7.1 Previous guidelines

This guideline replaces the previous guidelines of the NVMM on the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamases (ESBL) in Enterobacteriaceae [NVMM 2002, NVMM 2008]. Based on current knowledge the working group judged whether previous recommendations were still up to date and applicable to the Dutch situation. If appropriate, recommendations were revised or new recommendations were added.

1.7.2 Procedures working group

The current version of the guideline has been developed during a period of twelve months. Working group members systematically searched relevant literature and judged the quality and content of the retrieved publications. Subsequently, the working group members wrote chapters or paragraphs of the

guideline, assimilating the judged literature. The text of the guideline was discussed among members of the working group, and adjusted accordingly.

1.7.3 Method for guideline development

This guideline has been developed in accordance with the protocol 'Protocol voor de ontwikkeling, autorisatie en revisie van beroepsgebonden richtlijnen van de Nederlandse Vereniging voor Medische Microbiologie' that was issued by the NVMM in 2011 [NVMM 2011].
The recommendations in this guideline are, as much as possible, based on scientific insights from published studies, where both therapeutic and infection control aspects have been taken into account. If no data were available in the literature recommendations are based on expert opinion. In the event that recommendations are based on unpublished data, the specific data are provided in Appendix C.
The recommendations in this guideline are in accordance with the expert rules in antimicrobial susceptibility testing and the clinical breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), unless stated explicitly [EUCAST 2011, Leclerq 2011].

1.8 Authorisation and implementation

The final draft guideline will be provided to the NVMM for preconditional testing by it's Quality Committee, and subsequent comment by the users of the guideline. The working group will assimilate the comment provided by the users. The details of the comment round will be provided in Appendix D.
The final guideline will be provided to the assembly of the NVMM for authorisation. After authorisation the guideline will be distributed to all Dutch medical microbiology laboratories and clinical microbiologists. It will be published on the website and in the scientific journal of the NVMM.

1.9 Revision

Although this guideline reflects, as much as possible, the current knowledge on antimicrobial resistance, regular updating will remain necessary due to the ongoing technological developments and the emergence of new antimicrobial resistance mechanisms.
The working group is primarily responsible for the actuality of this guideline. By 2017, or earlier if deemed necessary, the working group will decide whether this guideline is still up to date or needs revision.

CHAPTER 2 - STAPHYLOCCUS AUREUS

2.1 Methicillin resistance

Dr. B.M.W. Diederen, Prof. dr. J.A.J.W. Kluytmans, Drs. M.F.Q. Kluytmans – van den Bergh, Prof. dr. C.M.J.E. Vandenbroucke – Grauls, Prof. dr. M.C. Vos, Prof. dr. A. Voss

2.1.1 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first described in 1961 [Jevons 1961]. MRSA produces an additional penicillin-binding protein, PBP2a [Hartman 1984, Reynolds 1985] that confers resistance to all currently available beta-lactam agents, including cephalosporins and carbapenems. PBP2a is encoded by the *mecA* gene [Matsuhashi 1986]. The expression of methicillin resistance in *S. aureus* is variable, resulting in heterogeneity of resistance and making detection of resistance often difficult [de Lencastre 1994, Montanari 1990]. *MecA* gene-negative *S. aureus* isolates with low-level methicillin resistance due to alterations of existing PBPs (referred to as 'moderately resistant *S. aureus*' (MODSA)) or hyperproduction of beta-lactamase (referred to as 'borderline oxacillin-resistant *S. aureus*' (BORSA)) have been described [Chambers 1989, de Lencastre 1991, McDougal 1986]. However, they are not frequently reported, and they are generally susceptible to other antimicrobial agents. Therefore, the Dutch 'search and destroy' policy for MRSA [Vandenbroucke 1998] does not include MODSA and BORSA. Consequently, in the laboratory detection of methicillin resistance, MODSA and BORSA should be distinguished from *S. aureus* containing the *mecA* gene.

2.1.2 Detection of carriage

2.1.2.1 Culture sites

MRSA screening cultures in both patients and health care workers should include a nasal swab (both anterior nares), a throat swab, and a perineal or rectal swab. The nose, more specifically the vestibulum nasi, is the most important carriage site of *S. aureus* [Kluytmans 1997, Matheson 2012, Vandenbergh 1999]. However, *S. aureus* can be isolated from other body sites, and not all carriers do carry *S. aureus* in the nose [Acton 2009, Lauderdale 2010]. A throat swab, added to a nasal swab, significantly increases the sensitivity of *S. aureus* detection in carriers and is considered necessary to reliably detect *S. aureus* carriage [Lauderdale 2010, Matheson 2012, Mertz 2007]. Also, perineal carriage of *S. aureus* is frequently observed and in some carriers the only site of carriage [Acton 2009, Coello 1994, Lauderdale 2010, Matheson 2012, Wertheim 2005]. Perineal and rectal swabs are considered comparable in their sensitivity to detect MRSA carriage [Acton 2009]. Recent studies suggest that groin samples may be used as an alternative to perineal swabs [Acton 2009, Lautenbach 2009, Senn 2012]. However, the working group considers the current evidence too limited to recommend groin samples as an alternative to the current standard, i.e. perineal or rectal samples.

Dependent on the clinical signs and age the following additional sites should be sampled:

Productive cough	-	sputum
Intubation	-	sputum or aspirate
Wound	-	wound swab

Other skin lesions, including eczema	-	skin swab
Indwelling urinary catheter	-	urine
Neonate	-	umbilical swab

Results from a recent prospective Dutch multicentre study indicate that culturing of insertion sites of intravascular catheters or drains has no additional benefit in the detection of MRSA carriage [Wassenberg *unpublished data*]. Since these samples require manipulation of the insertion site, which poses a potential risk to the patient, it is not recommended to culture insertion sites for the detection of MRSA carriage.

2.1.2.2 Number of cultures

A single set of specimens is considered sufficient for the targeted screening for MRSA carriage, provided that broth enrichment is used (see 2.1.3.3). Although repeated sampling may decrease the sample error, scientific data on this issue are currently insufficient to justify a recommendation to perform duplicate or repeated cultures. It is recommended to include a growth control in the work-up of specimens, and to repeat sampling of non-sterile culture sites if the growth control is negative (see 2.1.3.2).

Once a patient has been identified as a carrier of MRSA, it is not clear how many culture sets have to be taken to reliably identify loss of carriage. A recent Dutch study suggests taking at least five follow-up culture sets [Mollema 2010]. However, MRSA detection in this study was performed with a PCR method without conventional cultures, which is known to have reduced sensitivity. Therefore, the previous recommendation is maintained, i.e. patients that have been identified as MRSA carriers are considered to be at moderately elevated risk of persistent carriage (WIP risk category 3) when three culture sets, collected at least 7 days apart, and at least 48 hours after discontinuation of antibiotic therapy are negative. When an additional follow-up culture set taken after one year is still negative MRSA carriage is considered to be effectively cleared (WIP category 4) [WIP 2007]. Without treatment MRSA carriage may persist for months [Larsson 2011, Marschall 2006, Robicsek 2009, Sanford 1994, Scanvic 2001], in particular in patients that are hospitalised and use antibiotics. It is, therefore, not beneficial to take such follow-up cultures during hospitalisation in most cases.

2.1.2.3 Culture materials and transport

Staphylococci can survive for prolonged periods on dry surfaces and under various climatic conditions. Therefore, transport and storage conditions are not critical for detection. Nevertheless, it is recommended to use a transport medium (Amies or Stuart), as this has been shown to increase the detection rate when storage is prolonged [Riewerts Eriksen 1994]. The use of a dry swab is not recommended.

Recently, a new sampling system using a nylon-flocked swab in combination with a liquid transport medium, the Eswab, has been introduced. Because of its strong capillary activity the Eswab is supposed to provide a better sampling [van Horn 2008]. Several studies have reported increased detection rates for *S. aureus* when the Eswab was compared with conventional cotton tipped swabs

with transport medium [Saegeman 2011, Verhoeven 2010]. Others did not find a higher recovery rate [De Silva 2010, Smismans 2009]. Currently available data are considered insufficient to recommend the Eswab over conventional swabs with transport medium. It is recommended to process specimens as soon as possible, but at least within 24 hours after sampling, and keep samples at 4-8ºC until processing.

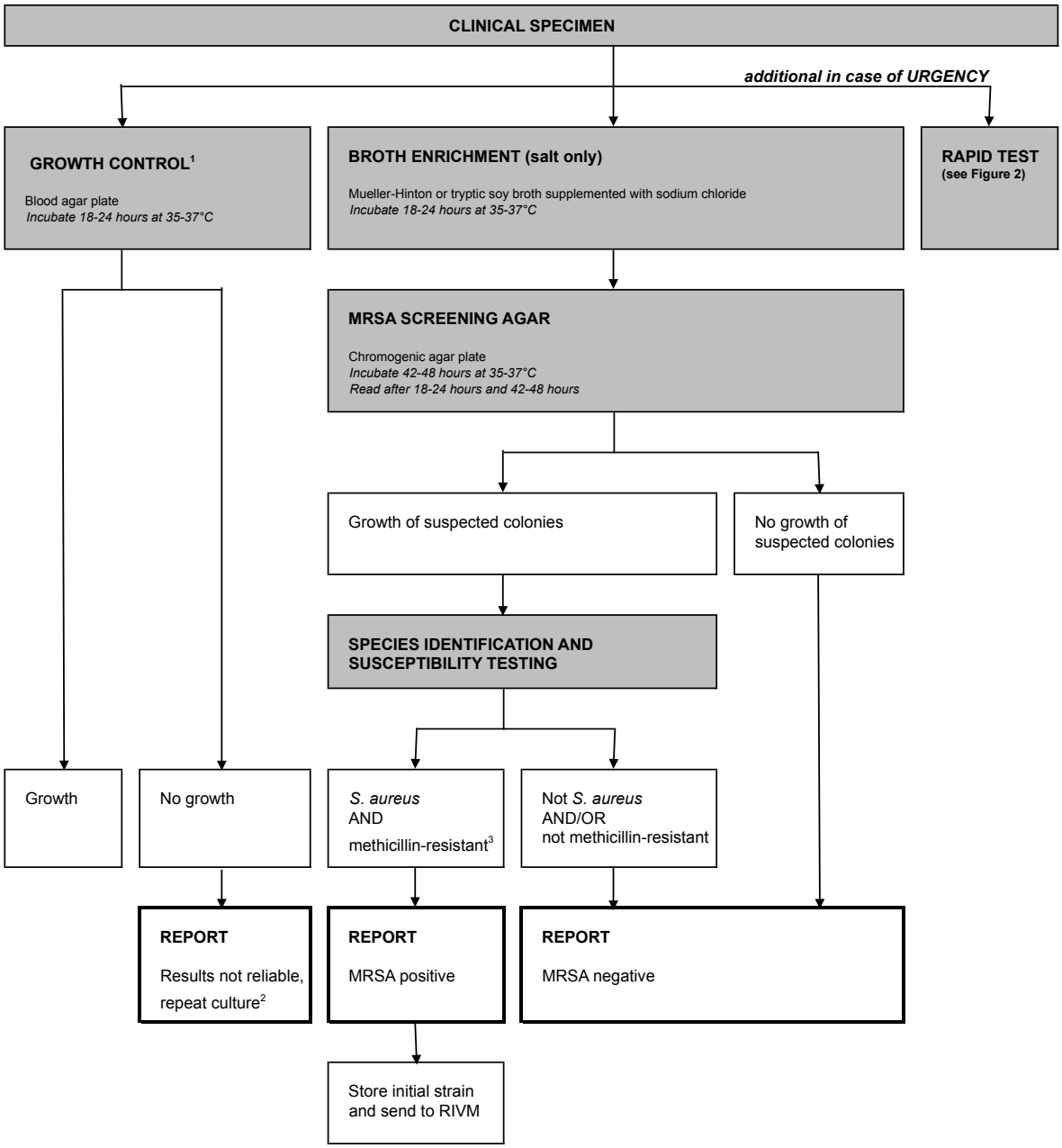
2.1.2.4 Timing of cultures in healthcare workers

Cultures for the detection of MRSA carriage in healthcare workers should be performed at the start of a working shift. Cultures that are taken during or at the end of a working day may detect transient MRSA carriage that will be cleared overnight. As this transient MRSA carriage does not contribute to sustained outbreaks it should preferably not be detected.

2.1.3 Laboratory methods

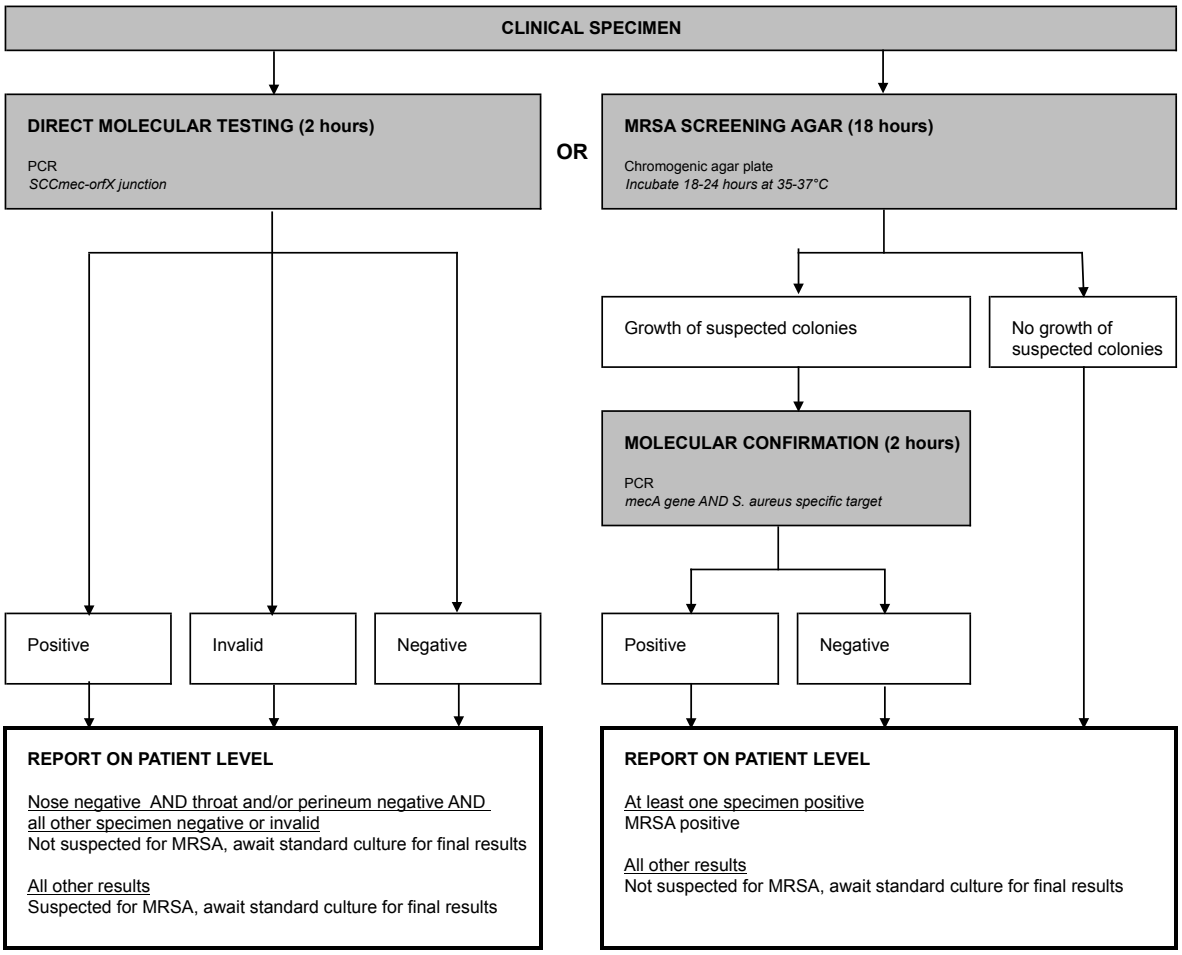
Several laboratory methods are recommended for the detection of methicillin resistance in *S. aureus*, including the use of an MRSA screening agar, relatively non-selective (salt only) broth enrichment and additional rapid methods in case of urgency (Figure 1 and Figure 2).

Figure 1. Laboratory methods for the targeted screening for MRSA carriage



1 May serve as a backup for MRSA isolates that are suppressed by the selective agents used in the MRSA screening agar
2 Non-sterile culture site only
3 If initial strain, confirm by molecular methods

Figure 2. Additional rapid laboratory methods, in case of urgency



2.1.3.1 Direct molecular detection

Direct molecular detection of MRSA in clinical samples can reduce the number of pre-emptive isolation days substantially [Aldeyab 2009, Harbarth 2006, Jeyratnam 2008A, Wassenberg 2010, Uçkay 2008]. It is most important that any direct molecular method should accurately distinguish between MRSA and mixtures of methicillin-susceptible *S. aureus* with methicillin-resistant coagulase-negative staphylococci in the sample. The SCC*mec-orfX* junction provides a unique target. It is important to realise that this region has a relatively high variability that may result in failure to detect some strains. On the other hand, SCC*mec* remnants in methicillin-susceptible (*mec*A gene negative) *S. aureus* may cause false-positive results [Lindqvuist 2012, Roisin 2012].

A recent Dutch multicentre study on rapid diagnostic tests for MRSA (n=1764 patients) included an evaluation of two MRSA PCR assays, the BD GeneOhm MRSA assay (BD Diagnostics) and the Xpert MRSA assay (Cepheid) [Wassenberg 2010]. The sensitivity to directly detect MRSA in clinical samples was reported to be 85% for BD GeneOhm MRSA and 75% for Xpert MRSA (reference method: conventional cultures with broth enrichment). For livestock associated MRSA the sensitivity was even lower, 70% and 68% respectively. The specificity was 97% for BD GeneOhm MRSA and 95% for Xpert MRSA. Other studies have reported similar findings [Jeyaratnam 2008B, Kelley 2009,

Rossney 2008, Wolk 2009]. The observed test characteristics in combination with the low prevalence of MRSA in the Netherlands result in a negative predictive value of 99% for both assays, but a relatively low positive predictive value of 44% and 33%, respectively. It is, therefore, recommended to only use direct molecular detection methods to provisionally exclude MRSA carriage. Both positive and negative test results should be confirmed by conventional cultures.

2.1.3.2 Solid agar media

Conventional media

The detection of MRSA from clinical specimens with non-selective conventional solid agar media may be hampered by overgrowth or the presence of populations with mixed susceptibilities. Nevertheless, it is recommended to use a conventional solid agar medium, such as a blood agar plate, as a growth control. MRSA cultures from non-sterile culture sites should be disapproved when the growth control is negative. In addition, the conventional medium may serve as a backup for MRSA isolates that are suppressed by the selective agents used in the MRSA screening agar. It is recommended to perform identification and susceptibility testing of morphologically suspected *S. aureus* colonies that grow on the conventional medium but not on the selective medium.

MRSA screening agar

For targeted MRSA screening of clinical specimens it is recommended to use an MRSA screening agar, as it allows rapid detection and isolation of MRSA. The use of chromogenic media has become a key method in the rapid identification of MRSA in clinical samples [Perry 2007], as it reduces the number of unnecessary isolation days and is cost saving [Wassenberg 2010]. The sensitivity of using a chromogenic agar (direct plating only and overnight reading) to detect MRSA in clinical samples was shown to be comparable to that of commercially available PCR based methods, using conventional cultures, including broth enrichment, as reference method [Wassenberg 2011].

Currently available chromogenic media for the detection of MRSA incorporate chromogens to differentiate *S. aureus* from other pathogens and antibiotics for selective growth of MRSA. Media differ in their chromogenic substrates and antibiotic formulations, and/or concentrations, factors that impact their sensitivity and specificity for the detection of MRSA [Malhotra 2008, Malhotra 2010, Verkade 2011]. Studies evaluating the performance of different chromogenic media have reported variable results. In general, specificities after 24 hours of incubation are almost uniformly high, whereas sensitivities tend to vary widely both between media and between studies [Luteijn 2011]. Prolonging the incubation time from 24-48 hours increases the sensitivity, but generally reduces the specificity [Luteijn 2011]. In a recent head-to-head comparison of five chromogenic media Brilliance MRSA agar (Oxoid) had the highest sensitivity (90%), but on the other hand the lowest specificity (87%) [Malhotra 2010]. The other four media, ChromID MRSA (bioMérieux), MRSASelect (Bio-Rad), CHROMagar MRSA (CHROM agar Microbiology) and BBL-CHROMagar MRSA (BD Diagnostics), had comparable sensitivities (81-83%), while the specificity was highest for CHROMagar (99%) and BBL-CHROMagar (99%). Based on the currently available data no recommendations for or against a specific product can be made. However, the recommended incubation time is 48 hours for either product. In case of

urgency molecular confirmation of suspected colonies can be performed after 18-24 hours of incubation (see 2.1.3.5 and 2.1.3.6). It is important to realise that the performance of selective media depends on the local epidemiology of MRSA, i.e. the relative frequency of variants of MRSA that are more or less suppressed by the selective agents used, and that specific MRSA strains may be missed by a selective approach.

2.1.3.3 Broth enrichment

The use of broth enrichment improves the performance of chromogenic solid media for the detection of MRSA in clinical samples [Böcher 2008, Böcher 2010, McAllister 2011, Verkade 2011]. A recent study has shown that the sensitivity of chromogenic media is not increased when samples are preincubated in a non-selective broth (75%) [Böcher 2010]. However, two other studies have shown that preincubation in a Mueller-Hinton- or trypticase soy broth supplemented with 6.5% sodium chloride significantly increased the sensitivity of chromogenic media (95 and 97% vs. 75% and 64%, respectively [McAllister 2011, Verkade 2011]. *In* vitro studies have shown that the salt tolerance of MRSA strains may vary, and that a broth containing > 2.5% sodium chloride may be inhibitory for some strains [Bruins 2007, Jones 1997]. Unfortunately, comparative data on the added value of preincubation of clinical samples in a broth containing 2.5% sodium chloride are not available. It should be noted that it is currently unclear whether a broth containing 2.5% sodium chloride would increase the sensitivity of chromogenic agar media to the same extent as a broth containing 6.5% sodium chloride. A sodium chloride concentration of 2.5% may be too low to sufficiently inhibit contaminating flora. The use of a relatively non-selective (salt only) broth limits the risk of suppression of certain MRSA variants, a phenomenon that was recently demonstrated for the previously recommended phenol red mannitol broth [Böcher 2008].

It has been suggested that preincubation of clinical samples before applying molecular methods would increase the yield of screening to the same extent as has been shown for chromogenic media. However, to our knowledge no head-to-head comparative data on the added value of broth enrichment prior to molecular detection are currently available. A recent study that compared the performance of molecular methods after preincubation of clinical samples reported a sensitivity of 94% compared with culture results using a selective phenol red mannitol broth [Kerremans 2008]. No comparison with direct molecular detection methods was made in this study. Moreover, a selective method was used as a reference method, which may have resulted in overestimation of the sensitivity. Besides these limitations, it should be noted that the added value of direct molecular detection methods, i.e. the potential to reduce the number of pre-emptive isolation days, disappears when samples are preincubated.

In conclusion, it is recommended to use relatively non-selective (salt only) broth enrichment in combination with the use of an MRSA screening agar for the detection of MRSA in clinical samples. The working group takes the view that there is insufficient evidence to currently recommend the use of broth enrichment in combination with molecular detection methods.

2.1.3.4 Pooling of samples

For patients it is recommended to use a separate swab for each culture site, and not to pool samples. First, the site of carriage may impact clinical decisions and treatment strategies [SWAB 2012]. Second, the effect of pooling of clinical samples on the diagnostic performance of conventional culture methods is insufficiently clear. Although reported *S. aureus* carriage rates are comparable for groups of individuals for whom samples are processed either pooled or separate [Mertz 2007], a direct comparison of pooled vs. separate processing of individual samples showed a reduced sensitivity (86%) for pooled processing [Grmek-Kosnik 2005]. For PCR based direct molecular detection methods, pooling of samples has been shown to increase the rate of inhibition and reduce the sensitivity [Jeyratnam 2008B, Kelley 2009, Svent-Kucina 2009, Wassenberg 2011]. Pooling of patient samples can be considered in case of an outbreak. For screening of healthcare workers it can be considered to use one swab to sample all three culture sites (nose, throat, and perineum), or to pool swabs. However, once MRSA carriage is detected all sites should be sampled separately before treatment is initiated, as the site of carriage determines the treatment strategy [SWAB 2012]. The decision to pool samples for cost-saving reasons should, therefore, always be weighed against the disadvantages of pooling.

2.1.3.5 Identification

Current routine identification methods for *S. aureus* should be used, as there are no indications that the identification of *S. aureus* is different for methicillin-susceptible or -resistant strains. Various methods can be used, including tube coagulase tests, latex agglutination tests, matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) or molecular methods. For newly detected MRSA carriers, it is recommended to confirm the species identification by molecular methods. Several commercial and in-house PCR based assays are available, including assays that simultaneously detect an *S. aureus*-specific target and methicillin resistance.

2.1.3.6 Susceptibility testing

The recommended strategy for the detection of methicillin resistance in *S. aureus* is a two-step procedure, and consists of a screening step followed by a genotypic confirmation step. The screening step is based on the reduced susceptibility of MRSA to oxacillin and cefoxitin. The genotypic confirmation step is based on the detection of the *mec*A gene. Phenotypic confirmation, based on the detection of PBP2a, may be performed, but should not be used as an alternative to genotypic confirmation. Recently, a variant of the *mec*A gene has been detected, called *mec*A_{LGA251}, which is not detected by conventional *mec*A PCR methods [Stegger 2012]. Strains that are oxacillin-resistant and are negative in the conventional *mec*A gene detection should be checked for the presence of this variant, either locally or at a reference centre. A multiplex PCR designed to differentiate *mec*A $_{LGA251}$ from the known *mec*A gene has recently been described [Stegger 2012].

Screening

- Routine susceptibility test methods

  Several routine susceptibility test methods can be used to screen for methicillin resistance in *S. aureus*, including broth dilution, agar dilution, or an automated system, such as VITEK 2 (bioMérieux) or Phoenix (Becton-Dickinson). Oxacillin is used as indicator beta-lactam as methicillin is no longer commercially available, and oxacillin maintains its activity during storage better. The recommended MIC screening breakpoint for oxacillin is > 2 mg/L [CLSI 2007, EUCAST 2012], corresponding with the EUCAST clinical breakpoint for 'oxacillin-susceptible' *S. aureus* (S: MIC ≤ 2 mg/L) [EUCAST 2012]. It is important to note that the variable expression of methicillin resistance *in vitro* (heteroresistance) may hamper the detection of resistance for some isolates [Bannerman 2003]. It is, therefore, recommended to use the cefoxitin disk diffusion method in addition to routine susceptibility test methods.
- Disk diffusion test

  For the disk diffusion test it is recommended to use cefoxitin as the indicator beta-lactam, as it is a better inducer of the *mec*A gene than oxacillin and disk diffusion tests using cefoxitin give clearer endpoints and are easier to read than tests with oxacillin [CLSI 2007, Skov 2003]. A Mueller-Hinton agar plate is inoculated with a bacterial suspension of 0.5 McFarland according to the manufacturer's instructions for use, and the cefoxitin disk (disk content 30 ug) is applied. It is recommended to incubate the Mueller-Hinton agar for 24 hours at 33-35ºC, because bacteria that express heteroresistance grow more slowly and may be missed at temperatures above 35ºC [CLSI 2007, Skov 2009]. The recommended zone diameter screening breakpoint for cefoxitin is < 22 mm [CLSI 2007, EUCAST 2012].

Confirmation – phenotypic

A rapid slide latex agglutination test based on the detection of PBP2a may be used for phenotypic confirmation of methicillin resistance. The method involves detection of agglutination with latex particles coated with monoclonal antibodies to PBP2a. The test is very sensitive and specific [van Griethuysen 1999, Sakoulas 2001, Smyth 2001]. Isolates producing small amounts of PBP2a may give weak or slow agglutination reactions. Rare isolates may give negative reactions.

Confirmation – genotypic

PCR based methods are the current standard for the detection of the *mec*A gene, that is considered the marker to detect MRSA. Detection of the *mec*A gene allows differentiation between MRSA and BORSA or MODSA, as the latter do not carry the *mec*A gene. Several commercial assays are available that successfully identify the *mec*A gene in purified cultures of *S. aureus*. All index strains of newly recognised MRSA carriers, i.e. patients, household members and healthcare workers, should be sent to the National Institute for Public Health and the Environment (RIVM) for genotypic confirmation of the presence of the *mec*A gene and genotyping.

Quality control

The following strains are recommended for quality control: *S. aureus* ATCC 33593 (methicillin-resistant); and *S. aureus* 29213 (methicillin-susceptible).

2.1.4 Contact tracing

2.1.4.1 Adjusting diagnostic methods in case of a 'known' strain

The culture sites to be sampled, and the processing of the specimen are similar to those specified for the targeted screening for MRSA carriage. It is essential, however, to confirm that the methods used for targeted screening are able to detect the 'known' strain. Dependent on the diagnostic characteristics of the 'known' strain, the methods used may be adjusted in order to improve the efficiency of MRSA detection.

2.1.4.2 Molecular typing

It is recommended to compare MRSA isolates that are detected in contact patients to the isolate of the index patient by (geno)typing of strains. Typing results are considered essential to recognize nosocomial transmission and to control outbreaks. Typing methods that can be used to identify transmission of MRSA include amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis (PFGE), ribotyping, *spa* typing, multilocus variable number tandem repeat analysis (MLVA), and Raman spectroscopy.

2.1.5 Reporting

2.1.5.1 Laboratory information system

In the laboratory system:

- Growth control results should be reported as either 'growth' or 'no growth'.
- Direct molecular test results should be reported as either 'MRSA-positive', 'invalid' or 'MRSA-negative'.
- Genotypic MRSA confirmation test results should be reported as either 'MRSA-positive' or 'MRSA-negative'.

2.1.5.2 Patient information system

Negative growth control

MRSA negative cultures from non-sterile culture sites with a negative growth control should be reported as 'results not reliable, repeat culture'.

Rapid test

Rapid test results (direct molecular testing or direct MRSA screening agar) should be reported on a patient level. Direct molecular test results should be reported as 'not suspected for MRSA, await standard culture for final results' if the nasal culture is negative, and either the throat culture or the perineal culture is negative, and all other sites have a negative or invalid test result. All other direct molecular test results should be reported as 'suspected for MRSA, await standard culture for final

results' [Diederen 2010, Wassenberg 2010]. Direct MRSA screening agar results should be reported as 'MRSA positive' if at least one clinical specimen has a positive test result. All other direct MRSA screening agar results should be reported as 'not suspected for MRSA, await standard culture for final results'. Results should be reported to the treating physician as soon as results are available to prevent delay in adequate treatment and infection control measures.

MRSA confirmation negative

MRSA-negative test results should be reported as 'MRSA-negative'. The antibiogram to be reported in the patient information system should be in accordance with the MICs determined for the antimicrobial agents tested, without further adjustments. Results should be reported to the treating physician as soon as results are available to prevent delay in adequate treatment and infection control measures.

MRSA confirmation positive

MRSA-positive test results should be reported as 'MRSA-positive'. The antibiogram to be reported in the patient information system should be in accordance with the EUCAST clinical breakpoints [EUCAST 2012]. However, isolates should be reported as resistant to all beta-lactam antibiotics, irrespective of the MICs measured [Leclerq 2011]. In addition, it is recommended to provide a warning that treatment should be performed in consultation with a clinical microbiologist or an infectious diseases consultant. Results should be reported to the treating physician as soon as results are available to prevent delay in adequate treatment and infection control measures.

2.1.6 Quality indicators

2.1.6.1 Laboratory turnaround time

Laboratory turnaround time (LTAT), i.e. the time from arrival of the clinical specimen in the laboratory until the time of (provisional or final) report [Breil 2011], has been selected as a quality indicator for the timely detection of the presence of MRSA. First, it is recommended to document LTAT (provisional and final) for all targeted MRSA screening cultures and for all clinical cultures that grow oxacillin/cefoxitin resistant *S. aureus.* Second, it is recommended to periodically monitor (provisional and final) LTAT for both MRSA-positive and MRSA-negative cultures. Recommended measures for monitoring are the mean, median, and 90th percentile of LTAT [Valenstein 1989]. Finally, it is recommended to monitor and investigate outliers of LTAT (e.g. >90th percentile) [Steindel 1999], in order to improve performance over time [Steindel 1997].

2.1.7 Recommendations

Detection of carriage

- MRSA screening cultures should include a nasal swab (both anterior nares), a throat swab, and a perineal or rectal swab.
- Dependent on the clinical signs and age additional sites should be sampled.
- It is not recommended to culture insertion sites for the detection of MRSA carriage.

- A single set of specimens is sufficient for the targeted screening for MRSA carriage, provided that broth enrichment is used.
- Cultures for the detection of MRSA carriage in healthcare workers should be performed at the start of a working shift.
- Patients that have been identified as MRSA carriers are considered to be at moderately elevated risk of persistent carriage (WIP risk category 3) when three culture sets, collected at least 7 days apart, and at least 48 hours after discontinuation of antibiotic therapy are negative. When an additional follow-up culture set taken after one year is still negative MRSA carriage is considered to be effectively cleared (WIP category 4).
- Swabs should be collected in an adequate transport medium (Amies or Stuart). The use of dry swabs is not recommended.
- It is recommended to process specimens as soon as possible but at least within 24 hours after sampling, and keep samples at 4-8ºC until processing.

Laboratory methods

- For targeted MRSA screening it is recommended to use relatively non-selective (salt only) broth enrichment in combination with the use of an MRSA screening agar.
- In case of urgency it is recommended to additionally perform rapid laboratory methods, such as direct molecular testing or direct plating of a MRSA screening agar.
- For chromogenic media the recommended incubation time is 48 hours.
- It is recommended to use a conventional solid agar medium as a growth control.
- MRSA cultures from non-sterile culture sites should be disapproved when the growth control is negative.
- It is recommended to perform identification and susceptibility testing of morphologically suspected *S. aureus* colonies that grow on the conventional medium but not on the selective medium.
- It is recommended to only use direct molecular detection methods to provisionally exclude MRSA carriage. Both positive and negative test results should be confirmed by conventional cultures.
- Direct molecular methods should accurately distinguish between MRSA and mixtures of methicillin-susceptible *S. aureus* with methicillin-resistant coagulase-negative staphylococci in the sample.
- For patients it is recommended to use a separate swab for each culture site, and not to pool samples.
- For screening of healthcare workers it can be considered to use one swab to sample all three culture sites (nose, throat, and perineum), or to pool swabs. Once MRSA carriage is detected all sites should be sampled separately before treatment is initiated.
- Current routine identification methods for *S. aureus* should be used, as there are no indications that the identification of *S. aureus* is different for methicillin-susceptible or -resistant strains.
- For newly detected MRSA carriers, it is recommended to confirm the species identification by molecular methods.

- The recommended strategy for the detection of methicillin resistance in *S. aureus* is a two-step procedure, and consists of a screening step followed by a genotypic confirmation step.
- Routine susceptibility test methods to screen for methicillin resistance are broth dilution, agar dilution, or an automated system.
- Oxacillin should be used as indicator beta-lactam in the screening for methicillin-resistance.
- The recommended MIC screening breakpoint for oxacillin is > 2 mg/L.
- It is recommended to use the cefoxitin disk diffusion method (incubation for 24 hours at 33-35ºC), in addition to routine susceptibility test methods.
- The recommended zone diameter screening breakpoint for cefoxitin is < 22 mm.
- *S. aureus* strains that are oxacillin-resistant and are negative in the conventional *mec*A gene detection should be tested for the presence of *mec*A_{LGA251}.
- PCR based methods should be used for the detection of the *mec*A gene.
- All index strains of newly recognised MRSA carriers should be sent to the National Institute for Public Health and the Environment (RIVM) for genotypic confirmation of the presence of the *mec*A gene and genotyping.
- The following strains are recommended for quality control: *S. aureus* ATCC 33593 (methicillin-resistant); and *S. aureus* 29213 (methicillin-susceptible).

Contact tracing

- For contact tracing it is recommended to confirm that the methods used for targeted screening are able to detect the 'known' strain.
- It is recommended to compare MRSA isolates that are detected in contact patients to the isolate of the index patient by (geno)typing of strains.

Reporting

- Growth control results should be reported in the laboratory system as either 'growth' or 'no growth'.
- Direct molecular test results should be reported in the laboratory system as either 'MRSA-positive', 'invalid' or 'MRSA-negative'.
- Genotypic MRSA confirmation test results should be reported in the laboratory system as either 'MRSA-positive' or 'MRSA-negative'.
- MRSA negative cultures from non-sterile culture sites with a negative growth control should be reported in the patient information system as 'results not reliable, repeat culture'.
- Rapid test results (direct molecular testing or direct MRSA screening agar) should be reported in the patient information system on a patient level.
- Direct molecular test results should be reported in the patient information system as 'not suspected for MRSA, await standard culture for final results' if the nasal culture is negative, and either the throat culture or the perineal culture is negative, and all other sites have a negative or invalid test result. All other direct molecular test results should be reported as 'suspected for MRSA, await standard culture for final results'.

- Direct MRSA screening agar results should be reported in the patient information system as 'MRSA positive' if at least one clinical specimen has a positive test result. All other direct MRSA screening agar results should be reported as 'not suspected for MRSA, await standard culture for final results'. Results should be reported to the treating physician as soon as results are available to prevent delay in adequate treatment and infection control measures.
- Confirmed MRSA-negative test results should be reported in the patient information system as 'MRSA-negative'. The antibiogram to be reported should be in accordance with the MICs determined for the antimicrobial agents tested, without further adjustments.
- Confirmed MRSA-positive test results should be reported in the patient information system as 'MRSA-positive'. The antibiogram to be reported should be in accordance with the EUCAST clinical breakpoints. However, isolates should be reported as resistant to all beta-lactam antibiotics, irrespective of the MICs measured. It is recommended to provide a warning that treatment should be performed in consultation with a clinical microbiologist or an infectious diseases consultant.
- MRSA test results should be reported to the treating physician as soon as results are available.

Quality indicators

- It is recommended to document LTAT (provisional and final) for all targeted MRSA screening cultures and for all clinical cultures that grow oxacillin/cefoxitin resistant *S. aureus.*
- It is recommended to periodically monitor (provisional and final) LTAT for both MRSA-positive and MRSA-negative cultures.
- Recommended measures for monitoring are the mean, median, and 90th percentile of LTAT.
- It is recommended to monitor and investigate outliers of LTAT.

CHAPTER 5 - ENTEROBACTERIACEAE

5.1 Extended-spectrum beta-lactamases

Dr. J. Cohen Stuart, Dr. M.A. Leverstein – van Hall, Dr. N. al Naiemi

5.1.1 Introduction

Extended-spectrum beta-lactamases (ESBLs) are defined as plasmid-encoded enzymes that are able to hydrolyze penicillins, oxyimino-cephalosporins of the 1st, 2nd and 3rd generation, and aztreonam [Paterson 2005]. ESBLs are not active against cephamycins and carbapenems, and are usually inhibited by beta-lactamase inhibitors such as clavulanic acid [Bradford 2001]. The most prevalent ESBLs belong to class A (TEM, SHV, CTX-M) [Paterson 2005]. The inhibitor-resistant class D (OXA) ESBLs are less prevalent [Naas 2008]. Occasionally other classes of ESBLs are detected [Lahey Clinic 2011]. The recent increase in the occurrence of ESBLs is largely due to the proliferation of CTX-M beta-lactamases [Livermore 2007, al Naiemi 2006, Paterson 2005].

The presence of AmpC beta-lactamases may interfere with the detection of ESBL [Paterson 2005, Stürenburg 2004]. Therefore, laboratory methods for the detection of ESBL will be discussed separately for those species in which inducible or derepressed chromosomal AmpC beta-lactamases are uncommon or absent (group I) and those for which the presence of inducible chromosomal AmpC beta-lactamases is more rule than exception (group II) (Table 1). It is important to note that AmpC beta-lactamases are increasingly found on plasmids that are species independent, and occur in both group I and group II Enterobacteriaceae [Navarro 2001, Voets 2011, Woodford 2007].

Table 1. Classification of Enterobacteriaceae according to the presence of chromosomal AmpC beta-lactamases

Group I[1]	Group II[2]
Escherichia coli	*Citrobacter freundii*
Klebsiella spp.	*Enterobacter* spp.
Proteus mirabilis	*Hafnia alvei*
Salmonella spp.	*Morganella morganii*
Shigella spp.	*Providencia* spp.
	Serratia spp.

1 Group I: Chromosomal AmpC beta-lactamases uncommon or absent
2 Group II: Chromosomal AmpC beta-lactamases common

5.1.2 Detection of carriage

5.1.2.1 Culture sites

Faeces or a rectal swab (visually contaminated) are the preferred specimens for the detection of carriage with highly resistant Enterobacteriaceae (HRE) [Paniagua 2010]. A perianal swab is slightly less sensitive, but is considered an acceptable non-invasive alternative to a rectal swab [Lautenbach 2005, Wiener-Well 2010]. A perineal swab is not recommended.

Dependent on the clinical signs and age the following additional sites should be sampled:

Productive cough - sputum

Intubation	-	sputum or aspirate
Wound	-	wound swab
Indwelling urinary catheter	-	urine
Neonate	-	throat swab

5.1.2.2 Number of cultures

A single set of specimens is considered sufficient for the targeted screening for carriage of HRE. Although repeated sampling may decrease the sample error, scientific data on this issue are currently insufficient to justify a recommendation to perform duplicate or repeated cultures.

Once a patient has been identified as a carrier of HRE, it is not clear how many culture sets have to be taken to reliably identify loss of carriage of HRE. Therefore, the working group has decided to follow the current recommendations for the detection of carriage of *Salmonella* spp. [Behravesh 2008], i.e. patients can be considered to be no longer carrying HRE if two culture sets, collected at least 24 hours apart, and at least 48 hours after discontinuation of antibiotic therapy are negative. Since carriage of HRE may be prolonged, in particular in patients that are hospitalised and use antibiotics [Hart 1982, Yagci 2009], the working group takes the view that it is not appropriate to take such follow-up cultures during hospitalisation.

5.1.2.3 Culture materials and transport

Swabs should be collected in an adequate transport medium that maintains the viability of the microorganisms without permitting rapid multiplication during transport. Stuart transport medium or Amies transport medium are recommended. The use of dry swabs is not recommended, as this is associated with a reduced yield [Moore 2007]. Specimens should be processed within 24 hours after sampling, and kept at 4-8ºC until processing.

5.1.3 Laboratory methods

5.1.3.1 Direct molecular detection

Standardised methods for the direct molecular detection of ESBL-producing Enterobacteriaceae (ESBL-E) in clinical samples are currently not available for routine use in medical microbiology laboratories.

5.1.3.2 Solid agar media

Conventional media

The detection of ESBL-E from clinical specimens with non-selective conventional media may be hampered by overgrowth or the presence of populations with mixed susceptibilities.

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ESBL screening agar

For targeted ESBL-E screening of clinical specimens it is recommended to use an ESBL-E screening agar, as it allows for rapid detection and isolation of ESBL-E. Three screening agars with good performance have been described in the literature: a selective agar, EbSA (Cepheid), and two

chromogenic agars, chromID ESBL (bioMérieux) and Brilliance ESBL (Oxoid) [Huang 2010, Overdevest 2011]. All three screening agars can be used with no major preference. However, for group II Enterobacteriaceae the EbSA agar has been shown to have a higher specificity than the chromID ESBL [Overdevest 2011].

5.1.3.3 Broth enrichment

To our knowledge only three studies are currently available that have evaluated the use of broth enrichment in the detection of ESBL-E. Although all three studies were relatively small and used different broth enrichment media, they all reported a higher yield when broth enrichment was used. One study reported a statistically significant better performance in spiked samples as well as in clinical samples [Murk 2009]. Two other studies both found a higher yield, although not statistically significant. [Diederen *unpublished data,* Kluytmans *unpublished data*]. At this point the working group takes the view that there is insufficient evidence to provide a firm recommendation on the use of broth enrichment for the detection of ESBL-E.

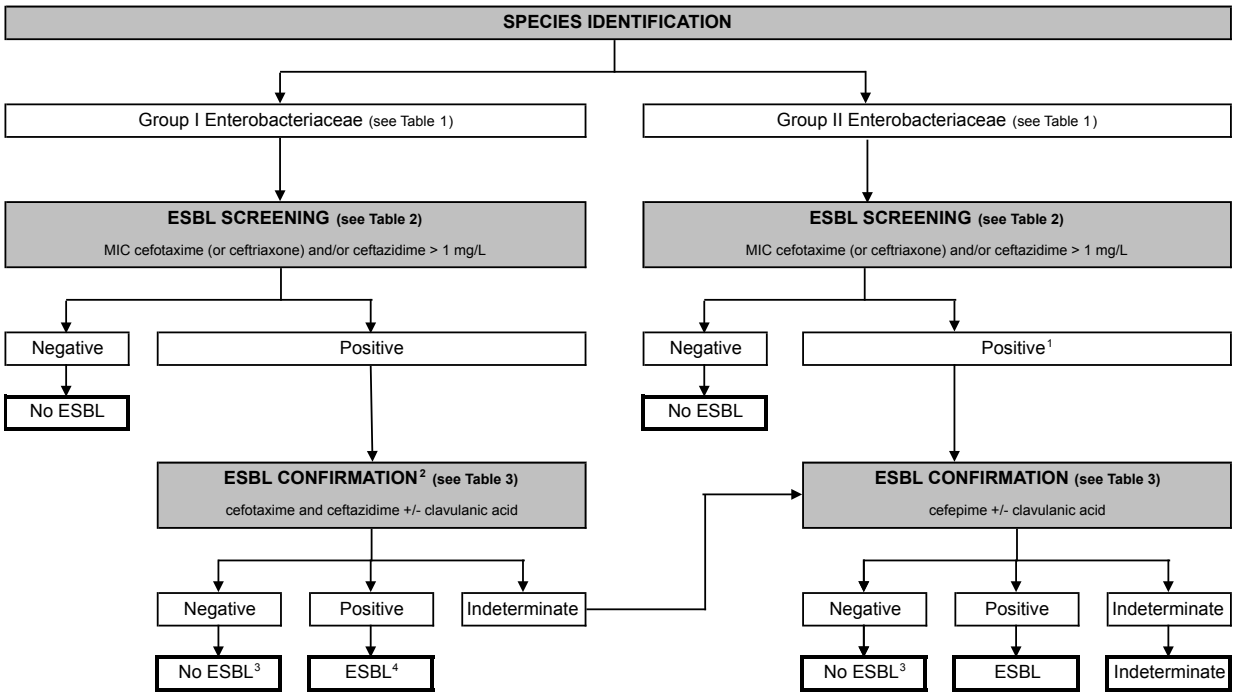
5.1.3.4 Identification

Current routine identification methods for Enterobacteriaceae should be used, as there are no indications that the identification of Enterobacteriaceae is different for susceptible or resistant isolates.

5.1.3.5 Susceptibility testing

The recommended strategy for the detection of ESBL in Enterobacteriaceae is a two-step procedure, and consists of a screening step followed by a confirmation step (Figure 1). The screening step is based on the reduced susceptibility of ESBL-producing isolates to indicator cephalosporins compared with isolates that belong to the wild type population. The confirmation step is based on the *in vitro* inhibition of ESBL activity by the addition of clavulanic acid (phenotypic confirmation) or the detection of ESBL resistance genes (genotypic confirmation). Screening alone is insufficient to reliably detect the presence of ESBL.

Figure 1. Algorithm for the detection of extended-spectrum beta-lactamases (ESBL) in Enterobacteriaceae



1 Derepressed chromosomosomal AmpC beta-lactamase gene may result in false-positive result.

2 If cefoxitin MIC ≥ 16 mg/L, than ESBL confirmation should additionally be performed with cefepime as indicator cephalosporin.

3 Inhibitor-resistant class D (OXA) ESBL can not be excluded.

4 Hyperproduction of K1 beta-lactamase in *Klebsiella oxytoca* may result in a false-positive result. A positive test result for ceftazidime is indicative of ESBL production.

Screening

A. Screening in group I Enterobacteriaceae

The recommended methods for ESBL screening in group I Enterobacteriaceae are broth dilution, agar dilution, disk diffusion or an automated system, such as VITEK 2 (bioMérieux) or Phoenix (Becton-Dickinson) (Table 2) [CLSI 2011, **Drieux 2008, Paterson 2005**]. It is recommended to use both cefotaxime (or ceftriaxone) and ceftazidime as indicator cephalosporins, as the MICs for cefotaxime (or ceftriaxone) and ceftazidime may differ for different types of ESBL [Biedenbach 2006, Hirakata 2005, Hope 2007, Kim 2004]. The use of cefpodoxime as indicator cephalosporin is not recommended. Although it is the most sensitive indicator cephalosporin to be used alone, it is less specific than the combination of cefotaxime (or ceftriaxone) and ceftazidime [Hope 2007]. For automated systems the combination of indicator cephalosporins for ESBL screening is dependent on the choice of the manufacturer, but should be in accordance with the recommendations on indicator cephalosporins provided in this guideline.

A screening breakpoint of > 1 mg/L is recommended for both cefotaxime (or ceftriaxone) and ceftazidime, in accordance with the guidelines issued by and CLSI (Table 2) [CLSI 2001, EUCAST 2012]. The screening breakpoints have been set to detect isolates with an MIC above the MIC distribution of the wild-type population. The recommended screening breakpoints correspond with the

EUCAST clinical breakpoints for 'susceptible' Enterobacteriaceae (S: MIC ≤ 1 mg/L) [EUCAST 2012], but are lower than the clinical breakpoint of the CLSI for ceftazidime (S: MIC ≤ 4) [CLSI 2011]. Corresponding zone diameters of indicator cephalosporins are shown in Table 2.

Table 2. ESBL screening methods for Enterobacteriaceae

Method	Antibiotic	Disk/tablet load	Screening positive if
Broth dilution	cefotaxime		MIC > 1 mg/L
	ceftazidime		MIC > 1 mg/L
Agar dilution	cefotaxime		MIC > 1 mg/L
	ceftazidime		MIC > 1 mg/L
Disk diffusion	cefotaxime	30 ug	Inhibition zone < 28 mm
	cefotaxime	5 ug	Inhibition zone < 21 mm
	ceftriaxone	30 ug	Inhibition zone < 23 mm
	ceftazidime	30 ug	Inhibition zone < 23 mm
	ceftazidime	10 ug	Inhibition zone < 22 mm
Automated systems	cefotaxime	n.a.	MIC > 1 mg/L
	ceftazidime	n.a.	MIC > 1 mg/L

MIC = minimal inhibitory concentration; n.a. = not applicable

References: [CLSI 2011, EUCAST 2012, Hope 2007, Leverstein-van Hall 2002, Spanu 2006, Thomson 2007]

B. Screening in group II Enterobacteriaceae

No recommendations on ESBL screening for group II Enterobacteriaceae are available in the international guidelines of the CLSI, the Health Protection Agency – British Society for Antimicrobial Chemotherapy (HPA-BSAC) or the Swedish Reference Group for Antibiotics (SRGA). For group II Enterobacteriaceae it is recommended to perform ESBL screening according to the methods described above for group I Enterobacteriaceae (Figure 1 and Table 2) [Paterson 2005]. However, the results of this screening will frequently be false-positive, due to derepression of the chromosomal AmpC gene in these species.

The use of VITEK 2 cefepime MICs is not recommended for ESBL screening in group II Enterobacteriaceae, as the sensitivity is only 54% [Cohen Stuart 2011]. Compared to screening based on MICs of cefotaxime and ceftazidime, the use of VITEK 2 Advanced Expert System (AES) ESBL alarm (bioMérieux) has been reported to increase the specificity of ESBL screening in group II Enterobacteriaceae (87% vs. 63%), but has a decreased sensitivity (92% vs. 100%) [Cohen Stuart 2011].

Confirmation – phenotypic

Several phenotypic methods based on the *in vitro* inhibition of ESBL activity by clavulanic acid are available for ESBL confirmation. However, three methods are recommended: 1) the combination disk diffusion test, 2) the Etest ESBL, or 3) broth microdilution (Table 3) [**Drieux 2008,** Jeong 2009, Paterson 2005]. However, the combination disk diffusion test showed a better specificity with comparable sensitivity, than the Etest ESBL [Platteel *unpublished data*].

The VITEK 2 ESBL confirmation test is not recommended for ESBL confirmation, based on the limited number of data and the diverging results that have been published [Leverstein-van Hall 2002, Spanu 2006, Thomson 2007]. It is recommended not to use the 'double disk approximation test', as its sensitivity is dependent on the optimal disk/tablet distance, and has been shown to be low in several studies [Bedenic 2007, Paterson 2005, Tzelepi 2000].

- Combination disk diffusion test

  A Mueller-Hinton agar or IsoSensitest agar plate is inoculated with a bacterial suspension of 0.5 McFarland according to the manufacturer's instructions for use, and the cephalosporin disks/tablets are applied. The inhibition zone around the cephalosporin disk/tablet combined with clavulanic acid is compared to the zone around the disk/tablet with the cephalosporin alone. The test is positive if the inhibition zone is ≥ 5 mm larger with clavulanic acid than without (and the isolate has an MIC > 1 mg/L for the cephalosporin tested, i.e. ESBL screening is positive) (Table 3) [CLSI 2011, HPA 2008, M'Zali 2000]. In all other cases the test result is negative.
- Antibiotic gradient on a strip method

  A Mueller-Hinton agar or IsoSensitest agar plate is inoculated with a bacterial suspension of 0.5 McFarland, the Etest ESBL strip (AB Biodisk) is applied, and the strip is read according to the manufacturer's instructions for use. The test is positive if a ≥ 8-fold reduction is observed in the MIC of the cephalosporin combined with clavulanic acid as compared to the MIC of the cephalosporin alone or if a deformation ellipse / phantom zone is present (and the isolate has an MIC > 1 mg/L of the cephalosporin tested, i.e. ESBL screening was positive) (Table 3). The test result is indeterminate if the strip cannot be read appropriately due to growth outside the range of the strip. In all other cases the test result is negative. The test is negative if the reduction in the MIC of the cephalosporin combined with clavulanic acid as compared to the MIC of the cephalosporin alone is less than 8-fold and/or if the Etest ESBL MIC of cefotaxime (or ceftriaxone) is < 0.5 mg/L and/or the Etest ESBL MIC of ceftazidime is < 1 mg/L (see manufacturer's instructions). The Etest ESBL MIC should be used for confirmation of ESBL production only; it is not reliable for determination of the MIC.
- Broth microdilution

  Broth microdilution is performed with Mueller-Hinton broth containing serial twofold dilutions of cefotaxime (or ceftriaxone), ceftazidime and cefepime at concentrations ranging from 0.25 to 512 mg/L, with and without clavulanic acid at a fixed concentration of 4 mg/L. A bacterial suspension is inoculated into each well of the microtiter plate [Jeong 2009]. The microtiter plate is incubated at 37°C for 18 to 24 hours. The test is positive if a ≥ 8-fold reduction is observed in the MIC of the

cephalosporin combined with clavulanic acid as compared to the MIC of the cephalosporin alone. In all other cases the test result is negative [Jeong 2009].

Table 3. ESBL confirmation methods for Enterobacteriaceae that are positive in the ESBL screening (see Table 2)

Group I Enterobacteriaceae (see Table 1)			
Method	**Antibiotic**	**Disk/tablet load**	**Confirmation is positive if**
Etest ESBL	Cefotaxime +/- clavulanic acid	-	MIC ratio[1] ≥ 8 or deformation ellipse / phantom zone present
	Ceftazidime +/- clavulanic acid	-	MIC ratio[1] ≥ 8 or deformation ellipse / phantom zone present
Combination disk diffusion test	Cefotaxime +/- clavulanic acid	Cefotaxime 30 ug Clavulanic acid 10 ug	≥ 5 mm increase in inhibition zone[2]
	Ceftazidime +/- clavulanic acid	Ceftazidime 30 ug Clavulanic acid 10 ug	≥ 5 mm increase in inhibition zone[2]
Broth microdilution	Cefotaxime +/- clavulanic acid	-	MIC ratio[1] ≥ 8
	Ceftazidime +/- clavulanic acid	-	MIC ratio[1] ≥ 8
	Cefepime +/- clavulanic acid	-	MIC ratio[1] ≥ 8
Group II Enterobacteriaceae (see Table 1)			
Method	**Antibiotic**		**Screening is positive if**
Etest ESBL *AB Biodisk*	Cefepime +/- clavulanic acid	-	MIC ratio[1] ≥ 8 or deformation ellipse / phantom zone present
Combination disk diffusion test	Cefepime +/- clavulanic acid	Cefepime 30 ug Clavulanic acid 10 ug	≥ 5 mm increase in inhibition zone[2]
Broth microdilution	Cefepime +/- clavulanic acid	-	MIC ratio[1] ≥ 8

ESBL = extended-spectrum beta-lactamase; MIC = minimal inhibitory concentration

1 MIC indicator cephalosporin / MIC indicator cephalosporin + clavulanic acid

2 Indicator cephalosporin + clavulanic acid compared with indicator cephalosporin alone

References: CLSI 2011, HPA 2006, Jeong 2009, M'Zali 2000, Paterson 2005, Stürenburg 2004

A. Phenotypic confirmation in group I Enterobacteriaceae

It is recommended to use both cefotaxime (or ceftriaxone) and ceftazidime for the confirmation of ESBL in group I Enterobacteriaceae (Table 3). Considering the varying affinity of the common classes of ESBL for cefotaxime and ceftazidime, synergy of clavulanic acid with at least one of these indicator cephalosporins is sufficient to confirm the presence of ESBL [CLSI 2011].

Indeterminate test results (Etest) and false-negative test results (combination disc diffusion test, Etest and broth microdilution) may result from the presence of AmpC beta-lactamases [Drieux 2008, Jacoby 2009, Munier 2010]. A cefoxitin MIC ≥ 16 mg/L is indicative for stable derepression of the AmpC beta-lactamase gene [Jacoby 2009]. Therefore, if test results for cefotaxime (or ceftriaxone) or ceftazidime are indeterminate (Etest) or when the isolate has a cefoxitin MIC ≥ 16 mg/L, it is recommended to perform an additional ESBL confirmation test using cefepime as indicator cephalosporin, as cefepime is not degraded by AmpC beta-lactamases [Drieux 2008]. In addition, indeterminate test results (Etest) may result from the presence of a carbapenemase gene [March 2010].

Other causes of false-negative test results are the presence of inhibitor-resistant class D (OXA) ESBLs [Naas 2008] or inhibitor-resistant TEM beta-lactamases [Sirot 1997].
ESBL confirmation tests that use cefotaxime as the indicator cephalosporin may be false-positive in *Klebsiella oxytoca* strains with hyperproduction of the chromosomal K1 beta-lactamase [Livermore 1995, Paterson 2005, Sanders 1996]. It is recommended to perform genotypic ESBL confirmation for *K. oxytoca* isolates that have a positive phenotypic ESBL confirmation test result.
Other, less common, causes of false-positive test results are hyperproduction of SHV-1 ESBLs in *Klebsiella pneumoniae* or the presence of class A-carbapenemases (including KPC) [Nordmann 2009, Wu 2001].

B. Phenotypic confirmation in group II Enterobacteriaceae
For group II Enterobacteriaceae it is recommended to perform ESBL confirmation tests with cefepime as the indicator cephalosporin (Table 3), as cefepime is not degraded by chromosomal AmpC beta-lactamases [Cohen Stuart 2011, Stürenburg 2004, Towne 2010]. Where the synergy between indicator cephalosporin and clavulanic acid may be masked in the presence of chromosomal AmpC beta-lactamases this will not occur when cefepime is used.

Confirmation – genotypic
For the genotypic confirmation of the presence of ESBL genes it is recommended to use PCR and ESBL gene sequencing [Bradford 2001] or a DNA microarray based method. The Check-KPC ESBL microarray (Check-Points) has recently been evaluated using different collections of selected ESBL-E containing the majority of known ESBL genes, and showed a good performance [Cohen Stuart 2010, Endimiani 2010, Naas 2010, Platteel 2011, Willemsen 2011]. Test results are obtained within 24 hours, which is more rapid than the phenotypic confirmation procedures. It should be noted that sporadically occurring ESBL and new ESBL genes are not detected by this microarray.

Quality control
The following strains are recommended for quality control: *K. pneumoniae* ATCC 700603 (ESBL-positive); and *E. coli* ATCC 25922 (ESBL-negative).

5.1.4 Contact tracing

5.1.4.1 Adjusting diagnostic methods in case of a 'known' strain
The culture sites to be sampled, and the processing of the specimen are similar to those specified for the targeted screening for carriage of ESBL-E. For contact tracing it is recommended to use a (selective) medium that is optimised to detect the 'known' strain (see *5.1.3.2*). It is essential to ensure that the 'known' strain grows in the medium that will be used for targeted screening.

5.1.4.2 Molecular typing
It is recommended to compare ESBL-E isolates that are detected in contact patients to the isolate of the index patient by performing routine species identification and subsequent (geno)typing of strains.

Nosocomial transmission of ESBL may occur on different levels: 1) transmission of ESBL-producing strains, 2) transmission of plasmids encoding for ESBL, and 3) transmission of ESBL resistance genes. Typing methods that can be used to identify transmission of ESBL-E include amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis (PFGE), ribotyping and Raman spectrosocopy. The analysis of potential transmission of plasmids and/or resistance genes goes beyond the scope of this guideline.

5.1.5 Reporting

5.1.5.1 Laboratory information system

ESBL confirmation test results should be reported in the laboratory information system as either 'ESBL-positive', 'ESBL-negative' or 'ESBL-indeterminate'.

5.1.5.2 Patient information system

ESBL confirmation negative

The antibiogram to be reported in the patient information system should be in accordance with the MICs determined for the antimicrobial agents tested, without further adjustments.

ESBL confirmation positive

The antibiogram to be reported in the patient information system should be in accordance with the EUCAST clinical breakpoints [EUCAST 2012]. However, different from what is stated in the EUCAST expert rules [Leclerq 2011], the working group takes the view that there is insufficient clinical evidence to support the treatment of infections with ESBL-E with non-carbapenem beta-lactam antibiotics. Thus, in case of an MIC below the clinical breakpoint for a beta-lactam antibiotic other than a carbapenem, it is recommended not to report the result for that particular antibiotic AND to provide a warning that it is unclear whether non-carbapenem beta-lactam antibiotics are effective in the treatment of serious infections caused by ESBL-E, and that treatment should be performed in consultation with a clinical microbiologist or an infectious diseases consultant.

ESBL confirmation indeterminate

The antibiogram to be reported in the patient information system should be in accordance with the EUCAST clinical breakpoints [EUCAST 2012]

5.1.6 Quality indicators

5.1.6.1 Laboratory turnaround time

Laboratory turnaround time (LTAT), i.e. the time from arrival of the clinical specimen in the laboratory until the time of report [Breil 2011], has been selected as a quality indicator for the timely detection of the presence of ESBL-E. First, it is recommended to document LTAT for all targeted ESBL screening cultures and for all clinical cultures that grow Enterobacteriaceae that are positive in the ESBL screening. Second, it is recommended to periodically monitor LTAT for both ESBL-E positive and ESBL-E negative cultures. Recommended measures for monitoring are the mean, median, and 90th

percentile of LTAT [Valenstein 1989]. Finally, it is recommended to monitor and investigate outliers of LTAT (e.g. >90th percentile) [Steindel 1999], in order to improve performance over time [Steindel 1997].

5.1.7 Recommendations

Detection of carriage

- Faeces or a rectal swab are the preferred specimens for the detection of carriage with HRE.
- Dependent on the clinical signs additional clinical sites should be sampled.
- A single set of specimens is sufficient for the targeted screening for carriage of HRE.
- Patients can be considered to be no longer carrying HRE if two culture sets, collected at least 24 hours apart, and at least 48 hours after discontinuation of antibiotic therapy are negative.
- Swabs should be collected in an adequate transport medium (Stuart or Amies). The use of dry swabs is not recommended.
- Clinical specimens should be processed within 24 hours after sampling, and kept at 4-8ºC until processing.

Laboratory methods

- For targeted ESBL-E screening of clinical specimens an ESBL-E screening agar should be used.
- Routine identification methods for Enterobacteriaceae should be used, as there are no indications that the identification of Enterobacteriaceae is different for susceptible or resistant isolates.
- Detection of ESBL in Enterobacteriaceae should be a two-step procedure, consisting of a screening step followed by a confirmation step.
- Methods for ESBL screening in Enterobacteriaceae are broth dilution, agar dilution, disk diffusion or an automated system.
- ESBL screening in Enterobacteriaceae should be performed with both cefotaxime (or ceftriaxone) and ceftazidime as indicator cephalosporins.
- The screening breakpoint is > 1 mg/L for both cefotaxime (or ceftriaxone) and ceftazidime,
- Phenotypic methods for ESBL confirmation are the combination disk diffusion test, the Etest ESBL, or broth microdilution.
- Phenotypic ESBL confirmation in group I Enterobacteriaceae should be performed with both cefotaxime (or ceftriaxone) and ceftazidime.
- In group I Enterobacteriaceae an additional ESBL confirmation test with cefepime as indicator cephalosporin is needed if test results for cefotaxime (or ceftriaxone) or ceftazidime are indeterminate, or when the isolate has a cefoxitin MIC ≥ 16 mg/L.
- Phenotypic ESBL confirmation in group II Enterobacteriaceae should be performed with cefepime.
- Genotypic ESBL confirmation should be performed for *K. oxytoca* isolates that have a positive phenotypic ESBL confirmation test result.
- Genotypic confirmation of the presence of ESBL genes can be performed by PCR and ESBL gene sequencing or a DNA microarray based method.

- The following strains are recommended for quality control: *K. pneumoniae* ATCC 700603 (ESBL-positive); and *E. coli* ATCC 25922 (ESBL-negative).

Contact tracing

- For contact tracing it is recommended to use a (selective) medium that is optimised to detect the 'known' strain.
- ESBL-E isolates that are detected in contact patients should be compared to the isolate of the index patient by performing routine species identification and subsequent (geno)typing of strains.

Reporting

- ESBL confirmation test results should be reported in the laboratory information system as either 'ESBL-positive', 'ESBL-negative' or 'ESBL-indeterminate'.
- The antibiogram to be reported in the patient information system should be in accordance with the EUCAST clinical breakpoints. However, in case of an MIC below the clinical breakpoint for a beta-lactam antibiotic other than a carbapenem, it is recommended not to report the result for that particular antibiotic AND to provide a warning that it is unclear whether non-carbapenem beta-lactam antibiotics are effective in the treatment of serious infections caused by ESBL-E, and that treatment should be performed in consultation with a clinical microbiologist or an infectious diseases consultant.

Quality indicators

- It is recommended to document LTAT for all targeted ESBL screening cultures and for all clinical cultures that grow Enterobacteriaceae that are positive in the ESBL screening.
- It is recommended to periodically monitor LTAT for both ESBL-E-positive and ESBL-E-negative cultures.
- Recommended measures for monitoring are the mean, median, and 90th percentile of LTAT.
- It is recommended to monitor and investigate outliers of LTAT.

CHAPTER 5 - ENTEROBACTERIACEAE

5.2 Carbapenemases

Dr. J. Cohen Stuart, Dr. M.A. Leverstein – van Hall, Dr. N. al Naiemi

5.2.1 Introduction

The rapid emergence and dissemination of Enterobacteriaceae that are resistant to carbapenems, such as imipenem and meropenem, poses a considerable threat to clinical patient care and public health. Carbapenemase-producing Enterobacteriaceae (CPE) are characterised by their resistance to virtually all beta-lactam antibiotics, including the cephalosporins and carbapenems. In addition, many of these strains are also resistant to fluoroquinolones, aminoglycosides and co-trimoxazole [Bratu 2005, Souli 2010]. Invasive infections with these strains are associated with high rates of morbidity and mortality [Bratu 2005, Souli 2010].

The carbapenemases fall into three classes according to their amino acid sequence: Ambler class A (serine carbapenemases); class B (metallo-carbapenemases) and class D (OXA carbapenemases) [Queenan 2007]. Within these classes, further divisions are made, and new variants are frequently encountered [Queenan 2007]. The rapid emergence and spread of CPE is mainly caused by epidemics of bacteria bearing plasmid-mediated KPC (class A), VIM-1 and NDM (class B), and OXA-48 (class D) enzymes. Carbapenem minimum inhibitory concentrations (MICs) observed in CPE can exhibit considerable variation depending on the type and expression of carbapenemase enzyme, the bacterial species, and the presence of other resistance mechanisms such as cephalosporinases (ESBL and AmpC), reduced permeability and/or efflux pumps [Falcone 2009, Pasteran 2009, Tenover 2006]. Increased carbapenem MICs in Enterobacteriaceae may also result from high expression of AmpC or CTX-M ESBLs in combination with porin alterations [Pasteran 2009, Woodford 2007].

5.2.2 Detection of carriage

5.2.2.1 Culture sites

Faeces or a rectal swab (visually contaminated) are the preferred specimens for the detection of carriage with highly resistant Enterobacteriaceae (HRE) [Paniagua 2010]. A perianal swab is slightly less sensitive, but is considered an acceptable non-invasive alternative to a rectal swab [Lautenbach 2005, Wiener-Well 2010]. A perineal swab is not recommended.

Dependent on the clinical signs and age the following additional sites should be sampled:

Productive cough	-	sputum
Intubation	-	sputum or aspirate
Wound	-	wound swab
Indwelling urinary catheter	-	urine
Neonate	-	throat swab

5.2.2.2 Number of cultures

A single set of specimens is considered sufficient for the targeted screening for carriage of HRE.

Although repeated sampling may decrease the sample error, scientific data on this issue are currently insufficient to justify a recommendation to perform duplicate or repeated cultures.
Once a patient has been identified as a carrier of HRE, it is not clear how many culture sets have to be taken to reliably identify loss of carriage of HRE. Therefore, the working group has decided to follow the current recommendations for the detection of carriage of *Salmonella* spp. [Behravesh 2008], i.e. patients can be considered to be no longer carrying HRE if two culture sets, collected at least 24 hours apart, and at least 48 hours after discontinuation of antibiotic therapy are negative. Since carriage of HRE may be prolonged, in particular in patients that are hospitalised and use antibiotics [Hart 1982, Yagci 2009], the working group takes the view that it is not appropriate to take such follow-up cultures during hospitalisation.

5.2.2.3 Culture materials and transport
Swabs should be collected in an adequate transport medium that maintains the viability of the microorganisms without permitting rapid multiplication during transport. Stuart transport medium or Amies transport medium are recommended. The use of dry swabs is not recommended, as this is associated with a reduced yield [Moore 2007]. Specimens should be processed within 24 hours after sampling, and kept at 4-8ºC until processing.

5.2.3 Laboratory methods

5.2.3.1 Direct molecular detection
Standardised methods for the direct molecular detection of CPE in clinical samples are currently not available for routine use in medical microbiology laboratories.

5.2.3.2 Solid agar media
Conventional media
The detection of CPE from clinical specimens with non-selective conventional media may be hampered by overgrowth or the presence of populations with mixed susceptibilities.

CPE screening agar
For targeted CPE screening of clinical specimens it is recommended to use a CPE screening agar, as it allows for rapid detection and isolation of CPE. At present, two chromogenic agars for the detection of CPE with good performance are available in the Netherlands: CHROMagar KPC (Chromagar Microbiology), a selective agar for the detection of carbapenemases [Adler 2011, Panagea 2011, Moran Gilad 2011, Samra 2008], and the Brilliance CRE agar (Oxoid) [Cohen Stuart, *unpublished data*]. In addition, selective agars for the detection of ESBLs, such as chromID ESBL (bioMérieux) and Brilliance ESBL (Oxoid) [Carrër 2010, Nordmann 2011] may also be used to detect CPE, although there have been anecdotal reports of OXA-48 producing isolates that do not co-express ESBL [Carrër 2010].

5.2.3.3 Broth enrichment

Current published data do not provide sufficient evidence to recommend the use of an enrichment broth for the detection of CPE.

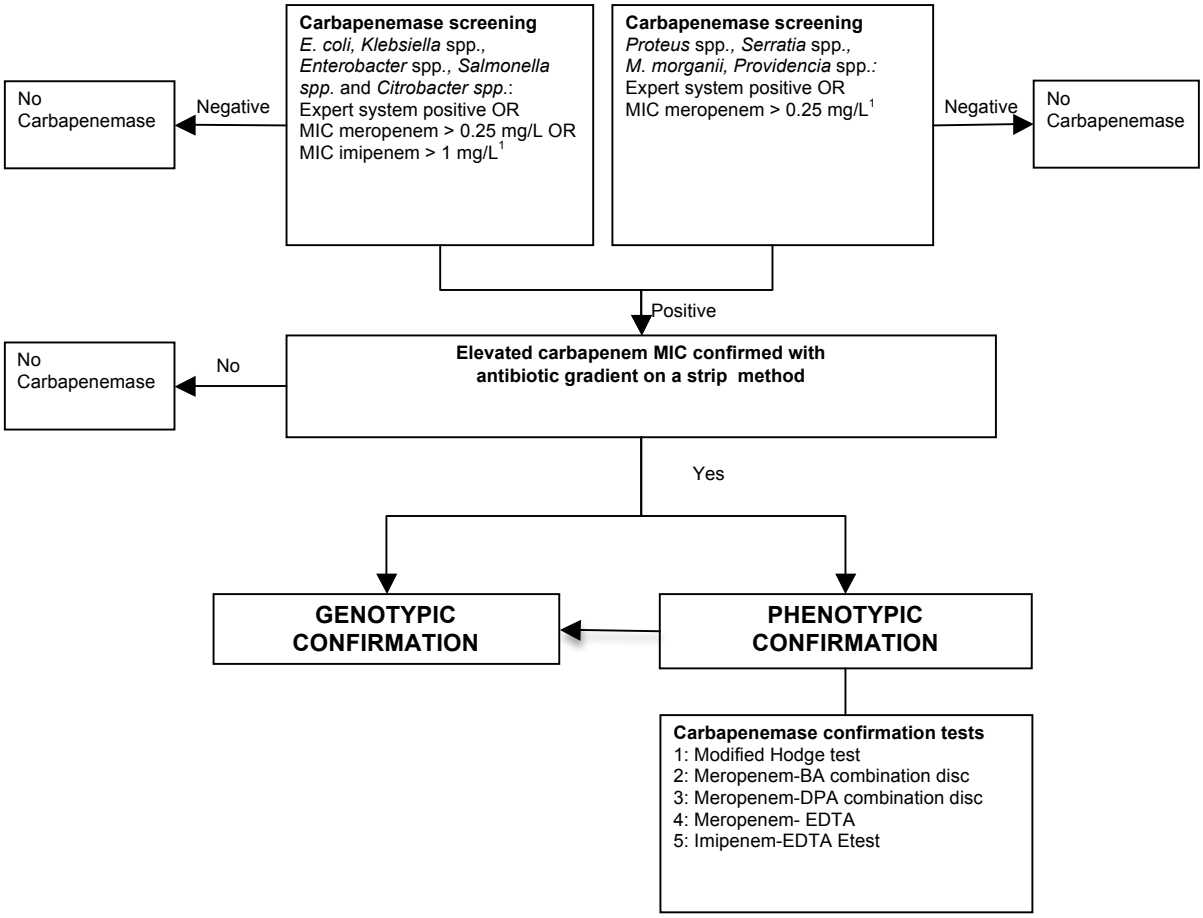
5.2.3.4 Identification

Current routine identification methods for Enterobacteriaceae should be used, as there are no indications that the identification of Enterobacteriaceae is different for susceptible or resistant isolates.

5.2.3.5 Susceptibility testing

The recommended strategy for the detection of carbapenemase production is a two-step procedure and consists of a screening step followed by a phenotypic and genotypic confirmation step (Figure 1). The screening step is based on the detection of reduced susceptibility to carbapenems in carbapenemase-producing isolates compared with isolates of the wild-type population. The phenotypic confirmation step is based on the detection of a diffusible carbapenemase and *in vitro* inhibition of carbapenemase activity upon addition of an inhibitor. The genotypic confirmation step consists of detection by polymerase chain reaction (PCR) and sequencing of carbapenemase genes.

Figure 1. Algorithm



BA = boronic acid derivative; DPA = dipicolinic acid; EDTA = ethylenediaminetetraacetic acid; MIC = minimal inhibitory concentration

1 The zone diameter screening breakpoint for meropenem has been set at < 24 mm with a disc content of 10 ug. For *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. the zone diameter screening breakpoint for imipenem has been set at < 22 mm with a disc content of 10 ug

Screening

Screening breakpoints

For each class of carbapenemases, and for each species and isolate, the MIC may vary from MICs of the wild-type population to > 256 mg/L, dependent on the presence of other resistance mechanisms. Setting of the recommended screening breakpoints has, therefore, been guided by the following principles: 1) the breakpoint MIC should be higher than the highest MIC of the wild-type population [EUCAST 2012], as the specificity of screening may otherwise become too low; and 2) the MIC breakpoint should be lower than the lowest carbapenem MICs described in the literature for strains shown to have a carbapenemase gene.

Meropenem - The recommended MIC screening breakpoint for meropenem has been set at > 0.25 mg/L for all Enterobacteriaceae, enabling the detection of the vast majority of carbapenemase-producers. Sporadic VIM-producers with meropenem MICs ≤ 0.25 mg/L and some OXA-48-producing

isolates will not be detected using this breakpoint [Falcone 2009, Poirel 2011]. The recommended zone diameter screening breakpoint for meropenem has been set at < 24 mm. Although this zone diameter breakpoint was shown to be less sensitive than the MIC screening breakpoint of > 0.25 mg/L (84%-97% vs. 100%, respectively) [Cohen Stuart *unpublished data,* Pasteran 2009], it was shown to detect all VIM- and KPC-producing isolates [Vading 2011].

Imipenem - For imipenem it is not possible to set a breakpoint for all Enterobacteriaceae, as some species (*Proteus* spp., *Serratia* spp., *Providencia* spp. and *Morganella morganii*) have a high imipenem MIC owing to mechanisms other than carbapenemase production [EUCAST 2012]. However, for pragmatic reasons and based on the available wild-type MIC distributions, a screening breakpoint has been set for those species for which the MIC of imipenem can be used. For *E. coli*, *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp. and *Citrobacter* spp. the recommended imipenem MIC screening breakpoint is > 1 mg/L, and the zone diameter screening breakpoint is < 22 mm. Although the recommended imipenem MIC screening breakpoint of > 1 mg/L was shown to have a sensitivity of 79% [Pasteran 2009], the breakpoint was not set lower because the MIC distribution of the wild-type population is up to 1 mg/L [EUCAST 2012]. It has been shown that the sensitivity of the imipenem zone diameter screening breakpoint of < 22 mm was 100% [Pasteran 2009, Vading 2011].

Ertapenem - Ertapenem is not advised as an indicator carbapenem in this guideline, since it has a lower specificity than imipenem and meropenem. Ertapenem is less specific because isolates with AmpC/ESBL and decreased permeability have higher MICs for ertapenem than for imipenem or meropenem [Cohen Stuart *unpublished data*, Woodford 2007]. However, in case of an outbreak with OXA-48 producing Enterobacteriaceae, it is recommended to use an ertapenem screening breakpoint of > 0.25 mg/L. OXA-48 producing Enterobacteriaceae may have a meropenem MIC ≤ 0.25 mg/L, whereas ertapenem MICs for these isolates are > 0.25 mg/L [Poirel 2011].

In conclusion - Carbapenemase screening in Enterobacteriaceae should be performed with both meropenem and imipenem. Routine screening with ertapenem is not recommended, but should be used in case of an outbreak with OXA-48 producing microorganisms.

Methods

Carbapenemase screening should be a standard component of the susceptibility testing on all Enterobacteriaceae isolated in routine diagnostics. This can take place by assessing the carbapenem MICs or by an alert from the expert system. When using automated systems for susceptibility testing (e.g. Phoenix, VITEK, or MicroScan), antibiotic panels containing both meropenem and imipenem are preferred. The preferred lowest concentration in the panels is 0.25 mg/L for meropenem, and 1 mg/L for imipenem.

The laboratory should be aware that strains with an MIC above the carbapenemase screening breakpoint but below the clinical breakpoint might nevertheless have a carbapenemase gene. Thus, strains with a meropenem MIC of 0.5 mg/L, 1 mg/L or 2 mg/L or an imipenem MIC of 2 mg/L are susceptible according to EUCAST clinical breakpoints, but should still be tested for the presence of a carbapenemase gene.

To exclude technical errors and to limit the number of strains to be confirmed for carbapenemase production, a carbapenem MIC above the screening breakpoint, measured by an automated system, should be confirmed by an antibiotic gradient on a strip method (e.g. Etest) with meropenem or imipenem on Mueller-Hinton agar (MHA) [Cohen Stuart 2010]. It is recommended not to use Iso-Sensitest agar as the carbapenem MICs of metallo-carbapenemase producers may be underestimated due to the low zinc concentrations [Walsh 2002]. Determining the MIC of carbapenemase-positive strains with an Etest can be complicated because mutant colonies with higher MICs than the dominant population may be found in the inhibition ellipse. These colonies should be included when interpreting the Etest, in accordance with the manufacturers' instructions.

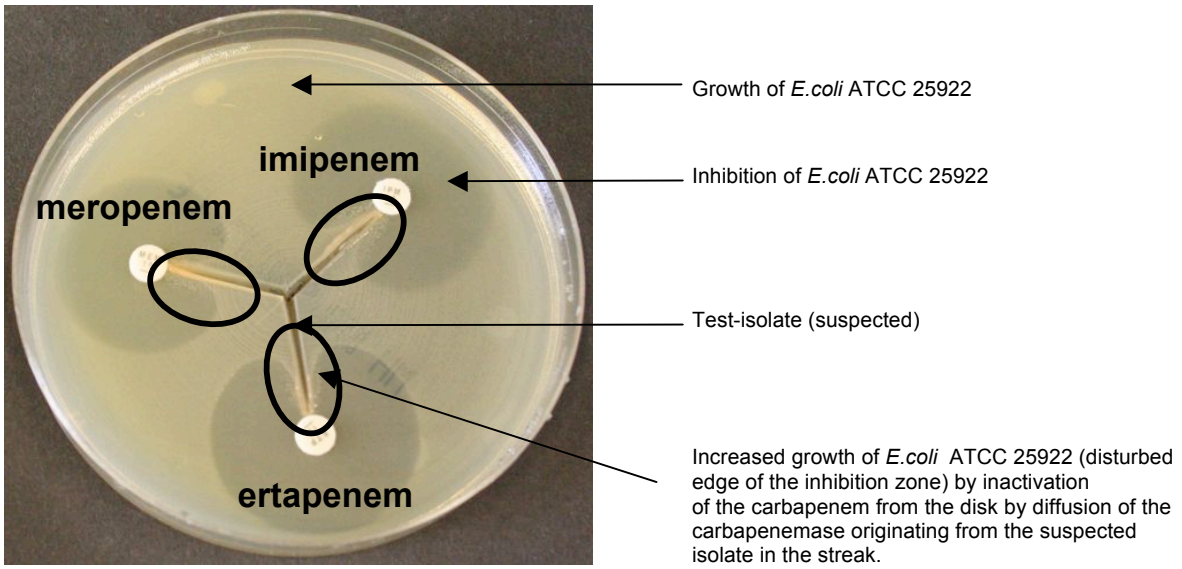
Confirmation – phenotypic

On the first isolate per species from a patient with a positive carbapenemase screen test, a PCR-based test should be performed to confirm the presence of carbapenemase genes (Figure 1). However, if genotypic confirmation is not immediately available, phenotypic confirmation tests can be performed in order to avoid delayed reporting of potential carbapenemase-producers to the clinic. Phenotypic confirmation can be performed using the modified Hodge test and/or the carbapenemase inhibition tests [Miriagou 2010, Pasteran 2009, Pasteran 2010].

Modified Hodge test

The modified Hodge test (Figure 2) is based on the detection of diffusible carbapenemases. It should be performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [CLSI 2010]. The modified Hodge test has a high sensitivity (95-100%) [Miriagou 2010, Pasteran 2009]. In addition, it is the only phenotypic confirmation test that is positive in case of OXA-48 production. Disadvantages of this test are the subjectivity and difficulty with test interpretation and the fact that different classes of carbapenemases cannot be distinguished. The specificity may be low because CTX-M ESBL- or AmpC beta-lactamase-producing isolates with reduced or absent porin expression may give false-positive results [Pasteran 2009, Pasteran 2010] (Table 2). However, for the detection of class A carbapenemases the specificity of the modified Hodge test can be increased by performing the double modified Hodge test [Pasteran 2010].

Figure 2. Modified Hodge test



Carbapenemase inhibition tests (synergy tests)

The carbapenemase inhibition tests are based on the *in vitro* inhibition of carbapenemase activity by addition of an inhibitor that is specific for a class of carbapenemases (resulting in a reduction in the MIC of the carbapenem). This phenomenon is called synergy between the carbapenem and the inhibitor. Carbapenemase inhibition tests can be used to distinguish between the different classes of carbapenemases (Table 1 and Table 2). For the detection of class A carbapenemases boronic acid (BA) derivatives are used as the inhibitor. To exclude AmpC as the cause of carbapenem resistance, an inhibition test with cloxacillin should be added to the boronic acid inhibition tests. For the detection of class B carbapenemases, ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid (DPA) can be used as an inhibitor.

It is recommended to use either combination disk diffusion tests or an antibiotic gradient on a strip method (e.g. Etest) with the strip containing both meropenem and an inhibitor. Table 1 shows the details of the combination tests as recommended by a group of experts from EUCAST and the ESCMID Study Group for Antibiotic Resistance Surveillance (ESGARS) [Miriagou 2010], as well as how these should be interpreted. Test characteristics and validation reports of the inhibition tests for the routine setting, only some of which are commercially available, are limited [Giske 2011, Tsakris 2010]. Double disk synergy tests (disk approximation methods) are not recommended, since the sensitivity depends on the optimal distance between the disks, which cannot be predicted [Bedenic 2007, Paterson 2005, Tzelepi 2000].

Table 1. Phenotypic confirmation methods for class A and class B carbapenemases

Carbapenemase	Method	Antibiotic	Disc/tablet load	Inoculum Medium	Confirmation is positive if
Class A	Combination diffusion - tablet *Rosco*	Meropenem +/- APBA	Meropenem 10 ug APBA	0.5 McF MHA	≥ 5 mm increase in inhibition zone[1]
	Combination diffusion - disc *In-house*	Meropenem +/- APBA	Meropenem 10 ug APBA 600 ug	0.5 McF MHA	≥ 4 mm increase in inhibition zone[1]
	Combination diffusion - disc *In-house*	Meropenem +/- PBA	Meropenem 10 ug PBA 400 ug	0.5 McF MHA	≥ 4 mm increase in inhibition zone[1]
Class B	Combination diffusion - disc *In-house*	Meropenem +/- EDTA	Meropenem 10 ug EDTA 292 ug	0.5 McF MHA[2]	≥ 5 mm increase in inhibition zone[1]
	Combination diffusion - disc *In-house*	Meropenem +/- EDTA	Meropenem 10 ug EDTA 730 ug	0.5 McF MHA[2]	≥ 5 mm increase in inhibition zone[1]
	Combination diffusion - tablet *Rosco*	Meropenem +/- DPA	Meropenem 10 ug DPA	0.5 McF MHA	≥ 5 mm increase in inhibition zone[1]
	Combination diffusion - tablet *In-house*	Meropenem +/- DPA	Meropenem 10 ug DPA 1000 ug	0.5 McF MHA	≥ 5 mm increase in inhibition zone[1]
	Etest MBL *AB Biodisk*	Imipenem +/- EDTA	-	0.5 McF MHA	MIC ratio[3] ≥ 8 or phantom zone present or deformation of ellipse[4]

APBA = 3-aminophenylboronic acid; DPA = dicolonic acid; EDTA = ethylenediaminetetraacetic acid; MBL = metallo-beta-lactamase; McF = McFarland standards; MHA = Mueller-Hinton agar; MIC = minimal inhibitory concentration; PBA = phenylboronic acid

1 Carbapenem + inhibitor compared with carbapenem alone

2 The brand of the MHA may influence the test characteristics of class B carbapenemase inhibition tests that use EDTA as inhibitor [Walsh 2002].

3 MIC imipenem / MIC imipenem + EDTA

4 To avoid false-negative results, the result of the MBL Etest should be interpreted as indeterminate if the MIC for imipenem < 4 mg/L AND the MIC for imipenem + EDTA < 1 mg/L.

References: [Giske 2011, Tsakris 2010]

Table 2. Interpretation of phenotypic carbapenemase confirmation test results

Confirmation test	Class of carbapenemase			AmpC beta-lactamase	ESBL
	Class A	*Class B*	*Class D*	with reduced permeability	with reduced permeability
Modified Hodge test					
Meropenem / imipenem	+	+	+	+/-	+/-
Carbapenemase inhibition tests					
Meropenem +/- APBA	+	-	-	+/-	-
Meropenem +/- PBA	+	-	-	+/-	-
Meropenem +/- DPA	-	+	-	-	-
Meropenem +/- cloxacillin	-	-	-	+/-	-
Meropenem +/- EDTA	-	+	-	-	-

APBA = 3-aminophenylboronic acid; DPA = dicolonic acid; EDTA = ethylenediaminetetraacetic acid; ESBL = extended-spectrum beta-lactamase; PBA = phenylboronic acid

Confirmation – genotypic

Genotypic confirmation comprises PCR detection and sequencing of carbapenemase genes. Alternatively, a microarray (e.g. Check-Points) may be used to detect the most prevalent carbapenemase genes (OXA-48, KPC, VIM, NDM and IMP) [Naas 2011]. The high diversity of genes with ever-increasing numbers of new variants implies that isolates with a negative genotypic result in the local laboratory setting should be sent to a reference laboratory for further genotypic confirmation. Currently, the following carbapenemase genes can be detected by PCR and sequencing: class A: KPC, SME, NMC-A, IMI, PER, GES, SFO, SFC, IBC; class B VIM, GIM, SIM, NDM, IMP, SPM; and class D: OXA, PSE [Dallenne 2010, Voets 2011].

Quality control

The following strains are recommended for quality control: *E. coli* ATCC 25922 (carbapenemase-negative); *K. pneumoniae* ATCC BAA-1705 (KPC-positive); and *K. pneumoniae* ATCC BAA-1706 (carbapenem-resistant due to other mechanisms than carbapenemase; modified Hodge test-negative).

5.2.4 Contact tracing

5.2.4.1 Adjusting diagnostic methods in case of a 'known' strain

The culture sites to be sampled, and the processing of the specimen are similar to those specified for the targeted screening for carriage of CPE. For contact tracing it is recommended to use a (selective) medium that is optimised to detect the 'known' strain (see *5.3.3.2*). It is essential to ensure that the 'known' strain grows in the medium that will be used for targeted screening.

5.2.4.2 Molecular typing

It is recommended to compare CPE isolates that are detected in contact patients to the isolate of the index patient by performing routine species identification and subsequent (geno)typing of strains. Nosocomial transmission of carbapenemase resistance may occur on different levels: 1) transmission of carbapenemase-producing strains, 2) transmission of plasmids encoding for carbapenem resistance, and 3) transmission of carbapenem resistance genes. Typing methods that can be used to identify transmission of carbapenemase-producing strains include amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis (PFGE), ribotyping and Raman spectroscopy. The analysis of potential transmission of plasmids and/or resistance genes goes beyond the scope of this guideline.

5.2.5 Reporting

5.2.5.1 Laboratory information system

Genotypic carbapenemase confirmation test results should be reported in the laboratory information system as either 'carbapenemase-positive', or 'carbapenemase-negative'.

5.2.5.2 Patient information system

CPE confirmation negative

The antibiogram to be reported in the patient information system should be in accordance with the MICs determined for the antimicrobial agents tested, without further adjustments.

CPE confirmation positive

The antibiogram to be reported in the patient information system should be in accordance with the EUCAST clinical breakpoints [EUCAST 2012]. However, different from what is stated in the EUCAST expert rules [Leclerq 2011], the working group takes the view that there is insufficient clinical evidence to support the treatment of infections with CPE with beta-lactam antibiotics. Thus, in case of an MIC below the clinical breakpoint for a beta-lactam antibiotic, it is recommended not to report the result for that particular antibiotic AND to provide a warning that it is unclear whether beta-lactam antibiotics are effective in the treatment of serious infections caused by CPE, and that treatment should be performed in consultation with a clinical microbiologist or an infectious diseases consultant.

5.2.6 Quality indicators

5.2.6.1 Laboratory turnaround time

Laboratory turnaround time (LTAT), i.e. the time from arrival of the clinical specimen in the laboratory until the time of report [Breil 2011], has been selected as a quality indicator for the timely detection of the presence of CPE. First, it is recommended to document LTAT for all targeted CPE screening cultures and for all clinical cultures that grow Enterobacteriaceae that are positive in the carbapenemase screening. Second, it is recommended to periodically monitor LTAT for both CPE-positive and CPE-negative cultures. Recommended measures for monitoring are the mean, median, and 90th percentile of LTAT [Valenstein 1989]. Finally, it is recommended to monitor and investigate outliers of LTAT (e.g. >90th percentile) [Steindel 1999], in order to improve performance over time [Steindel 1997].

5.2.7 Recommendations

Detection of carriage

- Faeces or a rectal swab are the preferred specimens for the detection of carriage with HRE.
- Dependent on the clinical signs additional clinical sites should be sampled.
- A single set of specimens is sufficient for the targeted screening for carriage of HRE.
- Patients can be considered to be no longer carrying HRE if two culture sets, collected at least 24 hours apart, and at least 48 hours after discontinuation of antibiotic therapy are negative.
- Swabs should be collected in an adequate transport medium (Stuart or Amies). The use of dry swabs is not recommended.
- Clinical specimens should be processed within 24 hours after sampling, and kept at 4-8ºC until processing.

Laboratory methods

- For targeted CPE screening of clinical specimens a CPE screening agar should be used. An ESBL-E screening agar may also be used, although OXA-48 producing isolates that do not produce ESBL cannot be detected.
- Routine identification methods for Enterobacteriaceae should be used, as there are no indications that the identification of Enterobacteriaceae is different for susceptible or resistant isolates.
- Detection of carbapenemase production in Enterobacteriaceae should be a two-step procedure, consisting of a screening step followed by a phenotypic and genotypic confirmation step.
- Carbapenemase screening in Enterobacteriaceae should be performed with both meropenem and imipenem. Routine screening with ertapenem is not recommended, but should be used in case of an outbreak with OXA-48 producing microorganisms.
- For all Enterobacteriaceae the MIC screening breakpoint for meropenem is > 0.25 mg/L, and the zone diameter screening breakpoint is < 24 mm.
- For *E. coli*, *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp., and *Citrobacter* spp. the MIC screening breakpoint for imipenem is > 1 mg/L, and the zone diameter screening breakpoint is < 22 mm.
- A carbapenem MIC above the screening breakpoint measured by an automated system should be confirmed with an antibiotic gradient on a strip method (e.g. Etest) on MHA (not on Iso-Sensitest).
- On the first isolate per species from a patient with a positive carbapenemase screen test, a PCR-based test should be performed to confirm the presence of carbapenemase genes.
- Phenotypic methods for CPE confirmation are the modified Hodge test and carbapenemase inhibition tests.
- The following strains are recommended for quality control: *E. coli* ATCC 25922 (carbapenemase-negative); *K. pneumoniae* ATCC BAA-1705 (KPC-positive); and *K. pneumoniae* ATCC BAA-1706 (carbapenem-resistant due to other mechanisms than carbapenemase; modified Hodge test-negative).

Contact tracing

- For contact tracing it is recommended to use a (selective) medium that is optimised to detect the ‘known’ strain.
- CPE isolates that are detected in contact patients should be compared to the isolate of the index patient by performing routine species identification and subsequent (geno)typing of strains.

Reporting

- Genotypic carbapenemase confirmation test results should be reported in the laboratory information system as either ‘carbapenemase-positive’, or ‘carbapenemase-negative’.
- The antibiogram to be reported in the patient information system should be in accordance with the EUCAST clinical breakpoints. However, in case of an MIC below the clinical breakpoint for a beta-lactam antibiotic, it is recommended not to report the result for that particular antibiotic AND to provide a warning that it is unclear whether beta-lactam antibiotics are effective in the treatment of

serious infections caused by CPE, and that treatment should be performed in consultation with a clinical microbiologist or an infectious diseases consultant.

Quality indicators

- It is recommended to document LTAT for all targeted CPE screening cultures and for all clinical cultures that grow Enterobacteriaceae that are positive in the carbapenemase screening.
- It is recommended to periodically monitor LTAT for both CPE-positive and CPE-negative cultures.
- Recommended measures for monitoring are the mean, median, and 90th percentile of LTAT.
- It is recommended to monitor and investigate outliers of LTAT.