Microbiological methods to detect intestinal carriage of highly-resistant microorganisms (HRMO) in humans and livestock in the i-4-1-Health Dutch-Belgian cross-border project

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Keywords

Antimicrobial resistance, intestinal carriage, detection, extended-spectrum beta-lactamase, carbapenem resistance, colistin resistance, ciprofloxacin resistance, vancomcin resistance, Enterobacterales, enterococci

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ABSTRACT

The i-4-1-Health project is a One Health project on the prevalence and spread of antimicrobial resistance in the human and veterinary domain in the Dutch-Belgian cross-border region. This paper describes the main components of the laboratory protocol that was developed to standardise the microbiological methods used for the detection of intestinal carriage of highly-resistant microorganisms.

INTRODUCTION

The global emergence and spread of antimicrobial resistance pose a critical threat to public health and livestock agriculture. The understanding and control of antimicrobial resistance is a complex global health challenge and asks for One Health approaches at both national and international levels.[1-4]
The i-4-1-Health project aims to address gaps in current knowledge and understanding of the complex routes of dissemination of antibiotic resistance by taking a One Health approach that includes both the human and veterinary domain and targets antibiotic use, infection control and antimicrobial resistance in the Dutch-Belgian cross-border region. Data are collected in hospitals, nursing homes, daycare centres, primary schools, and livestock farms. To the best of our knowledge, this is the first project to study intestinal carriage of a wide range of antimicrobial-resistant microorganisms in the human and veterinary domain in a cross-border setting.
This laboratory protocol was developed by the participating laboratories to standardise the microbiological methods used for the detection of intestinal carriage of highly-resistant microorganisms (HRMO), including extended-spectrum beta-lactamase-producing Enterobacterales (ESBL-E), carbapenem-resistant Gram-negative bacteria (carbaR-GNS), colistin-resistant Enterobacterales (coliR-E), ciprofloxacin-resistant Enterobacterales (ciproR-E) and vancomycin-resistant enterococci (VRE).

METHODS

Study period and setting

The i-4-1-Health study is performed in the Dutch-Belgian border region, comprising eight provinces (Figure 1). From October 2017 through January 2019, an anticipated number of 16,200 microbiological samples is collected in nine hospitals, 20 nursing homes, 20 daycare centres, 20 primary schools, 30 broiler farms and 30 multiplier pig farms in the Dutch-Belgian border region (Figure 2). Data are collected in cross-sectional surveys; three rounds of repeated surveys in hospitals, a single survey in nursing homes, daycare centres and primary schools, and three repeated surveys in livestock farms.

Laboratories

Microbiological cultures are processed in nine microbiology laboratories: 1) Laboratory of Medical Microbiology, Vaccine & Infectious Disease Institute, University of Antwerp, Antwerp, Belgium; 2) Laboratory of Clinical Microbiology, Antwerp University Hospital, Antwerp, Belgium; 3) Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium; 4) Department of Laboratory

Medicine, University Hospitals Leuven, Leuven, Belgium; 5) Microvida Laboratory for Medical Microbiology, Amphia Hospital, Breda, the Netherlands; 6) Laboratory for Microbiology, Admiraal De Ruyter Hospital, Goes, the Netherlands; 7) Department of Medical Microbiology, Maastricht University Medical Center+, Maastricht, the Netherlands; 8) Laboratory for Medical Microbiology and Immunology, Elisabeth-TweeSteden Hospital, Tilburg, the Netherlands; and 9) PAMM Laboratory for Pathology and Medical Microbiology, Veldhoven, the Netherlands.

Microbiological methods

Microbiological methods are standardised across the nine participating laboratories (Figure 3). Nylon-flocked swabs with 2 ml Cary-Blair medium (FecalSwab®, Copan Italy, Brescia, Italy) are used for collection and transport of faecal, perianal or gastrointestinal stoma samples. Swabs are kept at 2 to 8ºC, transported at room temperature and processed within 72 hours after sampling. Swabs that are not processed within 72 hours are rejected. A Columbia (5%) sheep blood agar plate (bioMérieux, Marcy l'Etoile, France) is inoculated with 10 ul of the Cary-Blair medium and serves as growth control. Swabs are rejected when the blood agar plate shows no bacterial growth, which is judged to be indicative of inappropriate sampling. The remaining Cary-Blair medium is used to inoculate 5 mL of a non-selective tryptic soy broth (TSB) (Copan Italy, Brescia, Italy). The blood agar plate and the TSB are incubated for 18 to 24 hours at 35 to 37°C. Subsequently, 10 ul of the TSB is subcultured on four commercially available selective agar plates, i.e. ChromID® ESBL, ChromID® CARBA, ChromID® OXA-48 and (for human samples) ChromID® VRE (bioMérieux, Marcy l'Etoile, France). In three laboratories (University of Antwerp, Antwerp University Hospital, Amphia Hospital), two additional *in-house* selective agar plates are used, i.e. McC ciprofloxacin, a MacConkey agar plate (Oxoid, Basingstoke, United Kingdom) with ciprofloxacin (2 mg/L; Sigma-Aldrich, Saint Louis, United States) and EMB colistin, an eosin methylene blue agar plate (Oxoid, Basingstoke, United Kingdom) with colistin (3.5 mg/L; Sigma-Aldrich, Saint Louis, United States), daptomycin (10 mg/L) and amphoterin B (5 mg/L).[5] Selective agar plates are incubated at 35 to 37°C for 18 to 24 hours, except for the ChromID® VRE which is incubated for 42 to 48 hours. The remaining TSB is stored at -80°C for future (molecular) analyses.

The methods used for microbial identification and antimicrobial susceptibility testing are dependent on local routine laboratory practice (Table 1). Automated mass spectrometry systems with MALDI-TOF technology are used for microbial identification, i.e. either the VITEK® MS system (bioMérieux, Marcy l'Etoile, France) or the MALDI Biotyper® (Bruker Daltonic, Bremen, Germany). Antimicrobial susceptibility testing is performed with the semi-automated systems VITEK® 2 (AST card N344 and P586) (bioMérieux, Marcy l'Etoile, France) or BD Phoenix™ (AST card NMIC-417 and PMIC-96) (Becton Dickinson, Sparks, MD, United States) or with the disc diffusion method (Neo-Sensitabs™, Rosco, Taastrup, Denmark; Bio-Rad®, Bio-Rad Laboratories, Marnes-la-Coquette, France; or BD BBL™ Sensi-Disc™, Becton Dickinson, Sparks, MD, United States) on Mueller Hinton E agar plates (MHA) (bioMérieux, Marcy l'Etoile, France) (Table 2 and Table 3). Reading of disc diffusion susceptibility tests is performed either manually or with an automated zone reader (SIRscan, i2a

Diagnostics, Montpellier, France; ProtoCOL 3, Synbiosis, Cambridge, United Kingdom; or AGADIO™, Bio-Rad Laboratories, Marnes-la-Coquette, France).

In addition, for Enterobacterales that grow on the EMB colistin agar plate, the MIC for colistin is determined with the MICRONAUT MIC-strip Colistin, a broth microdilution method with Mueller Hinton cation adjusted broth (Merlin Diagnostika, Bornheim, Germany).[6] For enterococci that grow on the ChromID® VRE, the MICs for vancomycin and teicoplanin are determined with the gradient test method ETEST® (bioMérieux, Marcy l'Etoile, France). MICs and disk diffusion inhibition zones are interpreted using EUCAST breakpoint tables, version 8.1.[7] For Enterobacterales that grow on the ChromID® ESBL, the production of ESBL is phenotypically confirmed by the combination disk diffusion method with cefotaxime (30 ug), ceftazidime (30 ug), and cefepime (30 ug), each alone and combined with clavulanic acid (10 ug) (Neo-Sensitabs™, Rosco, Taastrup, Denmark) on MHA plates (bioMérieux, Marcy l'Etoile, France). Test results are interpreted according to EUCAST guidelines for detection of resistance mechanisms (Table 4).[8] All antimicrobial-resistant bacterial isolates are stored at -80°C using cryobeads or a cryopreservative glycerol broth. An anticipated number of 2,600 highly-resistant bacterial isolates is selected for whole-genome sequencing to identify genetic markers for antimicrobial resistance, clonal relatedness of isolates within and between domains and horizontal transfer of resistance genes.

Quality control

The following isolates are used for quality control: *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 700603 (SHV-18), *K. pneumoniae* ATCC BAA1705 (KPC, ciprofloxacin resistant), *K. pneumoniae* NCTC 13442 (OXA-48), *E. coli* NCTC 13846 (MCR-1), *E. faecium* CCUG 36804 (vanA), and *E. faecalis* ATCC 51299 (vanB).

Data labelling

The labelling system is designed to ensure that data are collected, stored and accessible in a way that permits cross-border and cross-domain analyses. Unique study numbers are generated for each human and veterinary sample included in the study. Each study number contains unique numeric codes for 'country', 'domain', 'institution or farm', 'ward, group or stable', 'survey' and a serial number (Figure 4). The study number used for the collection of microbiological data is identical to that used for the collection of demographic and clinical data. Labels with study numbers are pre-printed to avoid errors of transcription and interpretation with hand-written labels. For each ward or stable, one sheet with all labels for the ward or stable is prepared.

Unique isolate numbers are generated for each antimicrobial-resistant bacterial isolate stored. Each isolate number contains a unique numeric code for 'laboratory' and a serial number (Figure 4).

Data storage

Microbiological culture results are recorded in the local laboratory information system. At regular intervals, data are transferred to the central database where the data undergo final cleaning and are prepared for analyses.

Ethics

For the human domain, the study protocol was reviewed by the Medical Research and Ethics Committee of the University Medical Center Utrecht, Utrecht, the Netherlands (Protocol Number 17-426/C), the Medical Research and Ethics Committee of the Maastricht University Medical Center+ (Maastricht, the Netherlands) (METC 2017-0115 and METC 2017-0116), the Ethics Committee of the University Hospital Antwerp, Antwerp, Belgium (Belgian Registration Number B300210733784), the Ethics Committee of the Ghent University Hospital, Ghent, Belgium (Belgian Registration Number B670201733428), and the Ethics Committee of the University Hospitals Leuven, Leuven, Belgium (S59580 BD1 and S61807). The study was judged to be beyond the scope of the Dutch Medical Research Involving Human Subjects Act and the Belgian Law on Experiments on Humans, dated May 7th, 2004. Written or verbal informed consent for data collection and taking a faecal, perianal or gastrointestinal stoma swab for microbiological culture is obtained from all participants or their legal representatives.

For the veterinary domain, approval by an animal welfare body is not required. The procedure to collect fresh faecal droppings is considered to cause no discomfort, and animals are neither handled nor sacrificed during the study (EC Directive 2010/63).

All human data are anonymised, i.e. data cannot be directly or indirectly related to their source. Data on institutions and farms are pseudonymised, i.e. identifying information is replaced by a code, and a key file that links this code to the identifying information is kept separate from the research data.

Data sharing

During the study, feedback of resistance data is provided in-between surveys in hospitals and livestock farms. All investigators will have access to the final datasets and bacterial isolate collections to the extent necessary for answering research questions that are included as such in the i-4-1-Health publication plan. The findings will be presented at national and international conferences and submitted to peer-reviewed journals for publication. Publications are anticipated from 2019 and onwards. No earlier than December 31st, 2020 and no later than December 31st, 2024, the final datasets and isolate collections will be made available in accordance with the FAIR (Findable, Accessible, Interoperable and Reusable) data principles.[9]

DISCUSSION

Strengths

- The i-4-1-Health project takes a One Health approach to address gaps in current knowledge and understanding of the complex routes of dissemination of antibiotic resistance.
- It is the first project to study intestinal carriage of a wide range of antimicrobial-resistant microorganisms in the human and veterinary domain in a cross-border setting using uniform methodology across domains.
- Repeated measurements in hospitals and livestock farms make it possible to study trends over time.

- The use of pre-enrichment and selective agar plates increases the sensitivity of the methods to detect intestinal carriage of HRMO.[10,11]

Limitations

- The human domain includes patients in hospitals, nursing home residents and children in primary school and daycare centres, but not adults in the general population.
- The veterinary domain includes broiler farms and multiplier pig farms, but not non-chicken poultry farms, finishing pig farms and veal farms.

ACKNOWLEDGEMENTS

We are grateful to the collaborators in the participating laboratories, hospitals, nursing homes, primary schools, daycare centres and livestock farms for their contribution to the collection of the microbiological and epidemiological data.

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Figure 1. The Dutch-Belgian border region.

This figure was kindly provided by Interreg Flanders-The Netherlands.

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Figure 2. Timeline and anticipated numbers for microbiological sampling in the participating hospitals, nursing homes, daycare centres, primary schools, broiler farms and pig farms.

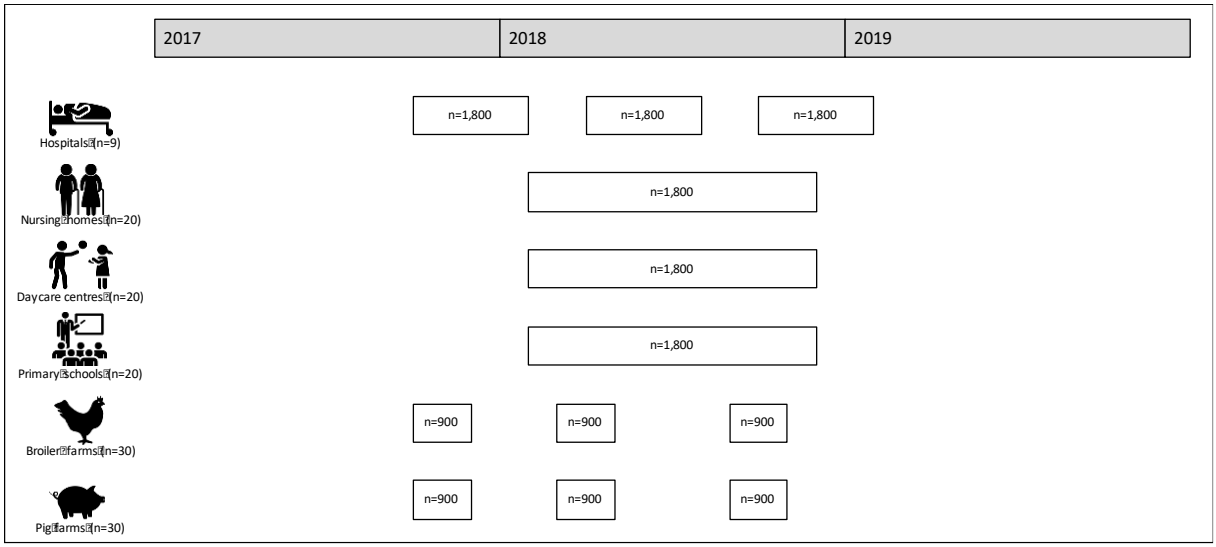

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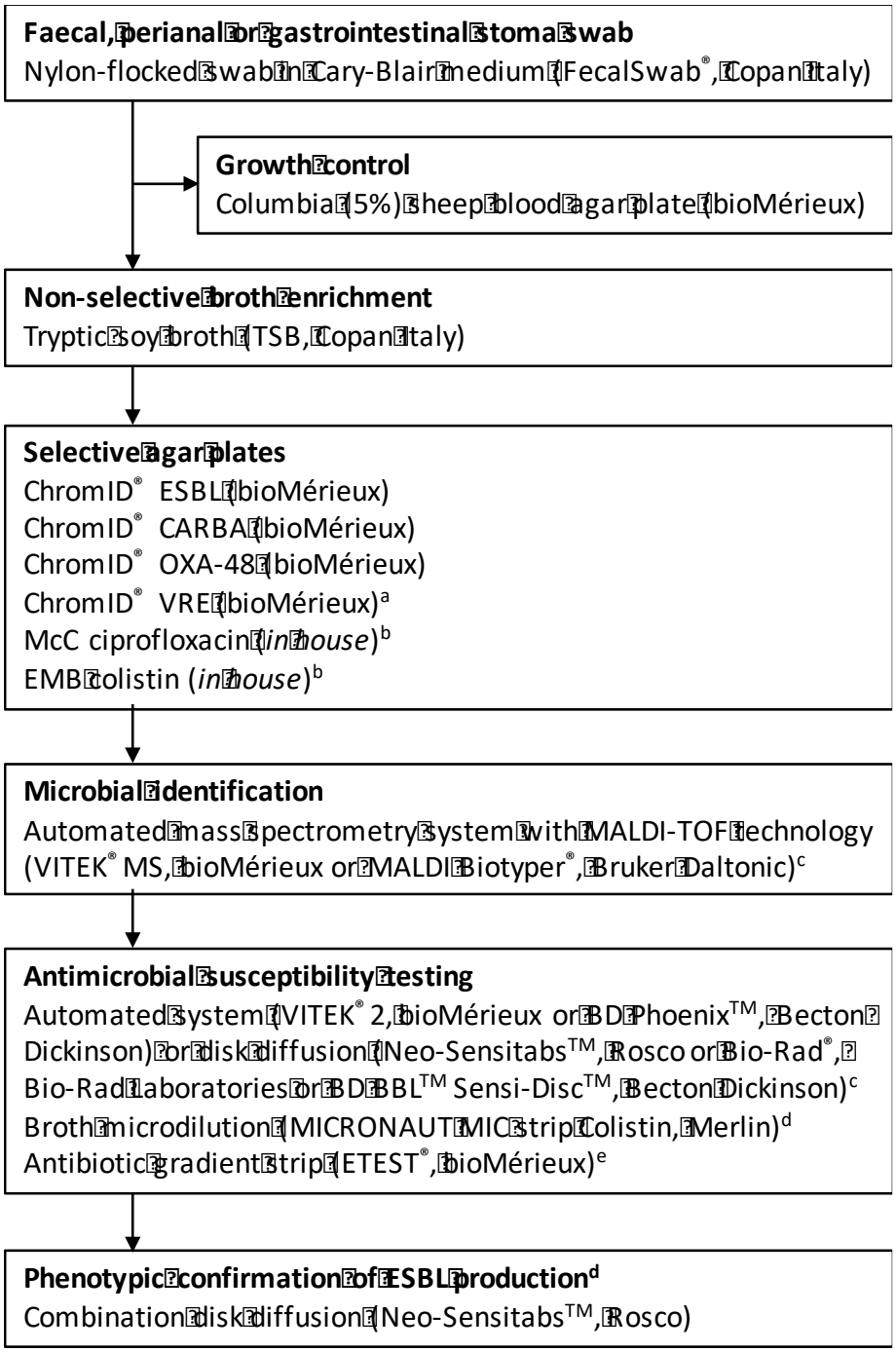
Figure 3. Flowchart of laboratory procedures.



a. ChromID® VRE are used for human samples only.

b. McC ciprofloxacin and EMB colistin are used in three of nine laboratories (Table 1).

c. The method used is dependent on local routine laboratory practice (Table 1).

d. Broth microdilution is used to determine the MIC for colistin in Enterobacterales.

e. The antibiotic gradient strip method is used to determine the MICs for vancomycin and teicoplanin in enterococci.

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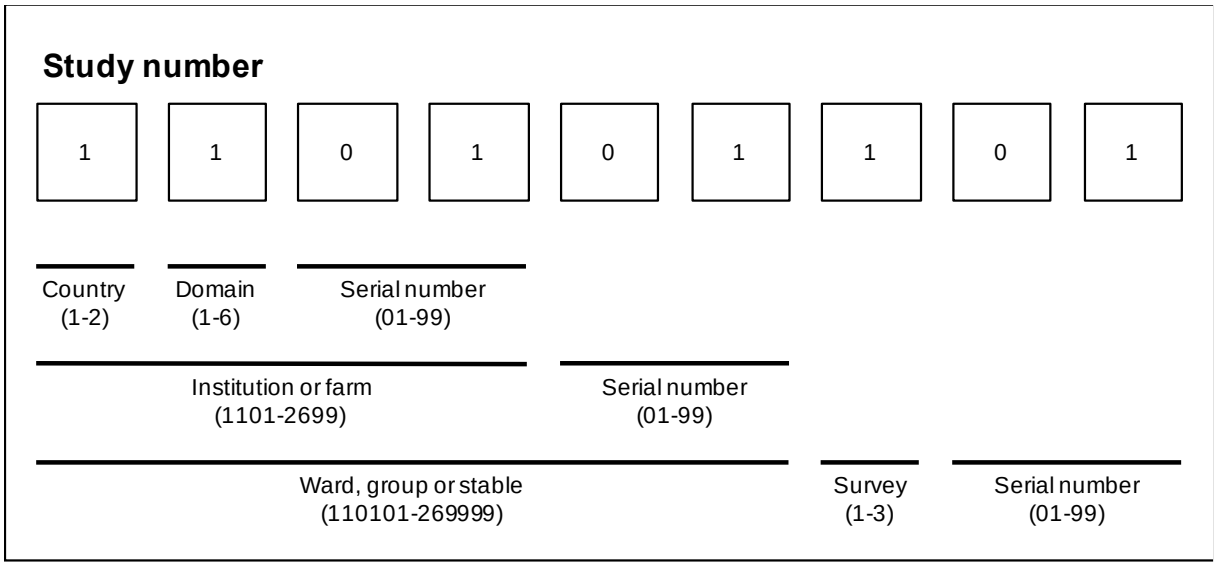
Figure 4. Structure of study numbers and isolate numbers.



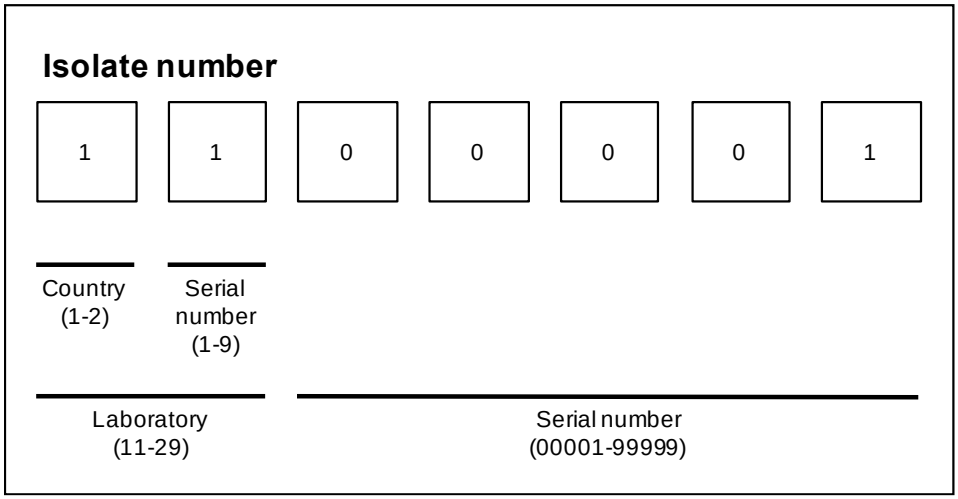


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Table 1. Laboratory methods used in the nine participating laboratories.

	Laboratory								
	ADRH	AH	AUH	ETH	GUH	MUMC	PAMM	UA	UHL
Faecal, perianal or gastrointestinal stoma swab									
FecalSwab® (Copan Italy)	x	x	x	x	x	x	x	x	x
Growth control									
Columbia (5%) sheep blood agar plate (bioMérieux)	x	x	x	x	x	x	x	x	x
Non-selective broth enrichment									
Tryptic soy broth (Copan Italy)	x	x	x	x	x	x	x	x	x
Selective agar plates									
ChromID® ESBL (bioMérieux)	x	x	x	x	x	x	x	x	x
ChromID® CARBA (bioMérieux)	x	x	x	x	x	x	x	x	x
ChromID® OXA-48 (bioMérieux)	x	x	x	x	x	x	x	x	x
ChromID® VRE (bioMérieux)[a]	x	x	x	x	x	x	x		x
McC ciprofloxacin (*in house*)		x	x					x	
EMB colistin (*in house)*		x	x					x	
Microbial identification									
VITEK® MS (bioMérieux)	x	x				x	x		
MALDI Biotyper® (Bruker Daltonic)			x	x	x			x	x
Antimicrobial susceptibility testing									
VITEK® 2 (bioMérieux)	x	x				x	x		x[c]
BD Phoenix™ (bioMérieux)				x					
Neo-Sensitabs™ (Rosco)			x[b]					x[b]	x[d]
Bio-Rad® (Bio-Rad Laboratories)					x[b]				
BD BBL™ Sensi-Disc™ (Becton Dickinson)			x					x	
MICRONAUT MIC strip Colistin (Merlin)[e]		x	x					x	
ETEST® (bioMérieux)[f]	x	x	x	x	x	x	x		x
Phenotypic confirmation of ESBL production									
Neo-Sensitabs™ (Rosco)	x	x	x	x	x	x	x	x	x

ADRH=Admiraal De Ruyter Hospital; AH=Amphia Hospital; AUH=Antwerp University Hospital; ETH=Elisabeth-TweeSteden Hospital; GUH=Ghent University Hospital; MUMC=Maastricht University Medical Center+; PAMM=PAMM Laboratory for Pathology and Medical Microbiology; UA=University of Antwerp; UHL=University Hospitals Leuven.

a. ChromID® VRE is used for human samples only.
b. For all isolates that are selected for whole genome sequencing, antimicrobial susceptibility testing with VITEK® 2 (bioMérieux) is additionally performed in Amphia Hospital.
c. For Enterobacterales, antimicrobial susceptibility testing is performed with VITEK® 2 (bioMérieux).
d. For enterococci, antimicrobial susceptibility testing is performed with Neo-Sensitabs™ (Rosco).
e. MICRONAUT MIC strip Colistin (Merlin) is used to determine the MIC for colistin in Enterobacterales.
f. ETEST® (bioMérieux) is used to determine the MICs for vancomycin and teicoplanin in enterococci.

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Table 2. Antimicrobial susceptibility testing in Enterobacterales.

	Automated systems		Disc diffusion method
	Vitek® 2 - N344 MIC range (mg/L)	BD Phoenix™ - NMIC-417 MIC range (mg/L)	Neo-Sensitabs™ / Bio-Rad® / BD-BBL™ Disc content (ug)
Amikacin		4–16	30
Amoxicillin/clavulanic acid	2/2–32/2	2/2–32/2	20/10
Ampicillin	2–32	2–8	10
Aztreonam			30
Cefepime		1–16	30
Cefotaxime	0.25–64		5
Cefoxitin	4–64	4–16	30
Ceftazidime	0.12–64	0.5–16	10
Ceftriaxone		0.5–4	30
Cefuroxime	1–64	2–8	30
Ciprofloxacin	0.25–4	0.25–1	5
Colistin	0.5–16	1–4	
Ertapenem		0.25–1	
Fosfomycin	16–256	16–128	200
Gentamicin	1–16	1–4	
Imipenem	0.25–16	0.25–8	
Levofloxacin		0.5–2	5
Meropenem	0.25–16	0.125–8	10
Nitrofurantoin	16–512	16–64	100
Norfloxacin		0.5–2	
Piperacillin/tazobactam	4/4–128/4	4/4–64/4	30/6
Temocillin			30
Tigecyclin		0.5–2	
Tobramycin	1–16	1–4	10
Trimethoprim	0.5–16	1–4	
Trimethoprim/sulfamethoxazole	1/19–16/304	1/19–4/76	1.25/23.75

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Table 3. Antimicrobial susceptibility testing in enterococci.

	Automated systems		Disc diffusion method
	Vitek®2 - P586 MIC range (mg/L)	BD Phoenix™ - PMIC-96 MIC range (mg/L)	Neo-Sensitabs™ / Bio-Rad® / BD-BBL™ Disc content (ug)
Ampicillin	2–32	1–16	2
Ampicillin/sulbactam	2/1–32/16		
Clindamycin	0.25–8		
Erythromycin	0.25–8		
Fosfomycin		16–64	200
Gentamycin high-level (synergy)	500	500	
Imipenem	1–16	1–8	
Levofloxacin		0.5–4	
Linezolid	0.5–8	0.5–4	10
Moxifloxacin	0.25–8		
Nitrofurantoin	16–512	16–64	
Quinupristin / dalfopristin	0.25–16		
Streptomycin high-level (synergy)	1000		
Teicoplanin	0.5–32	0.5–8	
Tetracyclin	1–16	0.5–2	
Tigecyclin	0.12–2	0.125–1	15
Trimethoprim		0.5–4	
Trimethoprim/sulfamethoxazole	0.5/9.5–16/304	0.5/9.5–4/76	
Vancomycin	0.5–32	0.5–8	5

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Table 4. Interpretation rules for the phenotypic confirmation of extended-spectrum beta-lactamase production in Enterobacterales.[8]

Enterobacterales	MIC cefoxitin (mg/L)	Combination disk diffusion - difference in inhibition zone			Conclusion
		CTX-C[c] - CTX30[d] (mm)	CAZ-C[e] - CAZ30[f] (mm)	FEP-C[g] - FEP30[h] (mm)	
Group 1[a]	-	>= 5	>= 5	-	ESBL positive
	-	>= 5	< 5	-	ESBL positive
	-	< 5	>= 5	-	ESBL positive
	>= 16	< 5	< 5	>= 5	ESBL positive
	< 16	< 5	< 5	>= 5	ESBL negative
	-	< 5	< 5	< 5	ESBL negative
Group 2[b]	-	-	-	>= 5	ESBL positive
	-	-	-	< 5	ESBL negative

a. Group 1 Enterobacterales (no inducible chromosomal AmpC) include *Citrobacter amalonaticus*, *Citrobacter farmeri*, *Citrobacter gillenii*, *Citrobacter koseri (diversus)*, *Citrobacter sedlakii*, *Escherichia coli*, *Escherichia fergusonii*, *Escherichia hermannii*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Kluyvera ascorbata*, *Leclercia adecarboxylata*, *Proteus mirabilis*, *Proteus penneri*, *Proteus vulgaris*, *Rahnella aquatilis*, *Raoultella* spp., *Salmonella* spp., and *Shigella* spp.

b. Group 2 Enterobacterales (inducible chromosomal AmpC) include *Citrobacter braakii*, *Citrobacter freundii*, *Citrobacter werkmanii*, *Citrobacter youngae*, *Citrobacter murliniae*, *Enterobacter cloacae (*complex*)*, *Hafnia alvei*, *Klebsiella aerogenes*, *Morganella morganii*, *Pantoea agglomerans*, *Providencia* spp., *Serratia* spp., and *Yersinia* spp.

c. Neo-Sensitabs™ CTX-C contains cefotaxime 30 ug and clavulanic acid 10 ug.

d. Neo-Sensitabs™ CTX30 contains cefotaxime 30 ug.

e. Neo-Sensitabs™ CAZ-C contains ceftazidime 30 ug and clavulanic acid 10 ug.

f. Neo-Sensitabs™ CAZ30 contains ceftazidime 30 ug.

g. Neo-Sensitabs™ FEP-C contains cefepime 30 ug and clavulanic acid 10 ug.

h. Neo-Sensitabs™ FEP30 contains cefepime 30 ug.