Transmission of antibiotic resistance genes via mobile genetic elements

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Definitions and abbreviations

Competence: *the ability of bacterial cells to take up extracellular ('naked') DNA from their surroundings by making use of a dedicated membrane system.*

Conjugation: *Transmission of foreign DNA via physical contact between donor and recipient bacterial cells.*

Conjugative plasmid: *Plasmids that can be transferred to other bacteria via conjugation and that carry all required genes for autonomous replication in the bacterial cells (origin of replication).*

Last resort antibiotics: *Antibiotics that are used as last options for treatments of infectious diseases when alternatives fail due to build-up of high (multiple) resistances to older antibiotics in pathogens, or for empiric use, e.g. in intensive care units.*

Lateral gene transfer (transmission): *Spread of genes to other bacteria, eventually crossing taxonomic borders.*

Mobile genetic element: *Mobile DNA structures in bacterial genomes responsible for intra- and intergenomic recombinations and/ or lateral transmission. Integrons, insertion (IS) elements, transposons, integrative conjugative elements (ICEs), phages and plasmids are mobile genetic elements.*

Mobilizable plasmid: *Conjugative plasmids that are transmitted with the help of another (self-transmissible) plasmid to other bacteria.*

Self-transmissible plasmid: *plasmid containing the complete set of genes that is required for transmission to other bacteria.*

Transformation: *Uptake of extracellular DNA by the cell, in the form of 'naked' DNA (i.e. unprotected by a cell wall or membrane), from its local environment.*

Transduction: *Transmission of foreign DNA from donor to recipient cells via bacteriophages.*

ARG	*Antibiotic resistance gene*
AME	*Aminoglycoside-modifying enzyme*
COGEM	*Commissie Genetische Modificatie*
ICE	*Integrative conjugative element*
LGT	*Lateral gene transfer*
LRA	*Last resort antibiotic*
MGE	*Mobile genetic element*
MIC	*Minimal inhibitory concentration*
MRSA	*methicillin-resistant Staphylococcus aureus*
PMQR	*plasmid-mediated quinolone resistances*
ST	*Sequence type*
WHO	*World Health Organization*

Voorwoord bij de resultaten van het COGEM project "Transmissie van antibioticumresistentie via mobiele genetische elementen".

Na de eerste introductie van antibiotica werd het voorkomen van resistentie tegen deze belangrijke geneesmiddelen bij bacteriesoorten al snel onderkend. Infecties die aanvankelijk effectief konden worden bestreden, bleken niet meer te reageren op antimicrobiële therapie, waardoor het risico op complicaties en sterfte weer toenam. De noodzaak ontstond om nieuwe antibiotica te ontdekken of oudere middelen te modificeren zodat de kans op onwerkzaamheid door resistentie zou verminderen. Aanvankelijk werden hiermee grote successen geboekt, maar telkens bleken enige tijd na introductie van een nieuw antibioticum bacteriën eigenschappen te ontwikkelen, waardoor zij toch weer resistent werden. Door toenemend gebruik van antibiotica bleek, met name in ziekenhuizen, maar ook daarbuiten, het resistentieprobleem in omvang toe te nemen.

Opgemerkt dient te worden dat antibioticumresistentie ook voorkomt in microorganismen in complexe ecosystemen, zoals in de bodem, waar van nature antibioticaproducerende bacteriën, gevoelige micro-organismen en schimmels in een ecologische balans voorkomen.

In de jaren zestig is, in eerste instantie door onderzoek in Japan, gebleken dat de genetische basis van resistentie niet alleen immobiel in de bacteriecel aanwezig is, maar dat deze zich ook kan bevinden op mobiele elementen. Deze mobiele elementen kunnen overgedragen worden naar bacteriestammen van de eigen soort en onder bepaalde voorwaarden zelfs aan andere soorten.

Wanneer een schadelijke ziekteverwekker beschikt over een verzameling van genen die coderen voor verschillende resistentiemechanismen, worden de mogelijkheden om een infectie te behandelen, verkleind en in het ergste geval onmogelijk. We spreken dan van een multiresistente ziekteverwekker. Bevinden de resistentiegenen zich ook nog in mobiele elementen, dan is er aanzienlijk risico dat onder de selectieve druk van antibiotica, met name in zorginstellingen, multiresistente bacteriën zich verspreiden en andere mensen en/of dieren besmetten.

Antibioticumresistentiegenen die de bestrijding van ziekteverwekkers in gevaar brengen, worden in de Regeling ggo bestempeld als een 'schadelijk genproduct'. De aanwezigheid van dergelijke antibioticumresistentiegenen in bacteriën waar ggo-werkzaamheden mee uitgevoerd worden, zou een reden kunnen zijn om deze werkzaamheden op een inperkingsniveau met strenge inperkingsmaatregelen in te schalen.

Eind 2017 is de COGEM gevraagd of de aanwezigheid van een antibioticumresistentiegen tegen vancomycine op een bacterieel plasmide tot een hogere inschaling van de betreffende bacterie zou moeten leiden. Om deze vraag te kunnen beantwoorden, heeft de COGEM opdracht gegeven tot een literatuuronderzoek naar de overdracht van antibioticumresistentiegenen die op mobiele elementen (zoals plasmiden) zijn gelegen.

Voor het uitvoeren van dit literatuuronderzoek is een beroep gedaan op de wetenschappelijke expertise van Dr. Leo van Overbeek. De begeleidingscommissie is samengesteld uit Prof. Dr. Ir. Jan Dirk van Elsas, Dr. Ir. Marjan Bovers, Dr. Ad Fluit en mijzelf. Het onderzoek is gestart in augustus 2018 en voltooid in maart 2019.

De hoeveelheid publicaties over antibioticumresistentie in de internationale literatuur loopt in de honderdduizenden. De auteur van het rapport heeft de zeer grote hoeveelheid publicaties na zorgvuldige selectie binnen een redelijke tijd geanalyseerd op informatie die van belang is voor de beantwoording van de onderzoeksvraag. De begeleidingscommissie is zich bewust van de tijdelijke houdbaarheid, omdat – ondanks dat hoofdzaken wetenschappelijk wel vast staan - de gedetailleerde wetenschappelijke kennis en de praktische mogelijkheden op dit specifieke gebied aan snelle veranderingen onderhevig zijn.

Prof. Dr. John Degener

(voorzitter begeleidingscommissie)

Samenvatting

Antibioticum resistenties in bacteriën kunnen ‘intrinsiek’ zijn, dat wil zeggen dat het aangrijpingspunt van het antibioticum ontbreekt in de cel of dat het antibioticum het aangrijpingspunt in de cel niet kan bereiken, of ‘verkregen’, en dan is de resistentie veroorzaakt door mutaties in het genoom of overgedragen vanuit andere bacteriën. Met het oog op de risico’s zijn alleen overdraagbare antibioticumresistentiegenen relevant in de context van dit rapport. Mobiele genetische elementen (MGEs) spelen een belangrijke rol bij de overdracht van genen die resistenties veroorzaken tegen antibiotica. Eventuele associatie van antibioticumresistentiegenen (ARGs) met MGEs in elke bacteriesoort duidt op het feit dat deze ARGs via laterale genoverdracht (LGO) zijn verkregen. Het doel van dit rapport is dan ook om aspecten aan te duiden die relevant zijn bij laterale overdracht van ARGs, met name van genen die betrokken zijn bij resistenties tegen laatste redmiddel antibiotica (LRA), en de MGEs waarop deze genen zijn gelokaliseerd in bacteriële genomen met de mogelijkheden tot LGO in het laboratorium.

De wereldgezondheidsorganisatie (WHO) lijst van kritisch relevante antimicrobiële middelen (https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/cia/en/) is in dit rapport gebruikt als richtlijn om het begrip LRA te definiëren. Deze groep van LRA omvat derde, vierde en vijfde generaties van cefalosporinen en verder carbapenems, glycopeptiden, macroliden en ketoliden, polymyxines, quinolonen, aminoglycosiden, ansamycinen, glycylcyclines en aminomethylcyclines, lipopeptiden en oxazolidinonen. De meeste aandacht in dit rapport is gericht op de meest recent ontwikkelde en goedgekeurde agentia binnen iedere antimicrobiële klasse, en in mindere mate op de ‘oudere’ antimicrobiële middelen die al sinds tientallen jaren worden voorgeschreven voor medische en diergeneeskundige toepassingen. Een “semi-systematische” literatuurzoekopdracht is uitgevoerd waarbij er in eerste instantie via automatische zoekopdrachten referenties zijn verzameld uit literatuurdatabestanden, waarna met de hand verder is geselecteerd op basis van relevantie binnen de kaderstelling van dit rapport. De zoekopdracht werd uitgevoerd in vijf verschillende literatuurdatabestanden en omvatte de termen ‘ antibiotic resistance’ alleen, of in combinatie met ‘mobilome’, ‘resistome’, ‘microbiome’, ‘mobile genetic element’, ‘pangenome’, ‘gene’, ‘self-transmissible’ en/ of ‘host range’. De geselecteerde referenties zijn vervolgens gebruikt voor verdere screening op verwijzingen naar ‘last resort antibiotic’. Op basis van gegevens uit de aldus geselecteerde referenties bleek dat genen die resistenties

veroorzaken tegen LRA, dikwijls gekoppeld zijn aan MGEs in zowel klinische als omgevingsbacteriën.

Deze ARGs zijn vaak afkomstig van andere bacteriën, en zijn verkregen via LGO. De diversiteit van MGEs, waarmee deze ARGs gekoppeld zijn, was groot, uiteenlopend van verschillende typen integrons (gelokaliseerd op transposons of plasmiden), insertie (IS) elementen, transposons, integratieve conjugatieve elementen (ICEs), plasmiden en in enkele gevallen ook (pro)fagen. Genen die resistentie veroorzaken tegen LRA blijken in sommige gevallen ook gelokaliseerd te zijn op chromosomen. De kans op laterale overdracht van chromosomaal gecodeerde ARGs, in afwezigheid van MGEs, wordt als verwaarloosbaar klein beschouwd. MGEs spelen een belangrijke rol bij de overdracht van ARGs naar andere bacteriesoorten.

Twee stappen zijn belangrijk bij het mobiliseren en overdraagbaar maken van ARGs, en dat zijn: 1) ‘rekrutering’ waarbij resistentiegenen opnieuw worden gerangschikt waardoor deze onder controle van alternatieve regulatie systemen komen te vallen, en dat gebeurt voornamelijk door (super) integrons, en 2) ‘klustering’ waarbij verschillende resistentiegenen tezamen worden gelokaliseerd op hetzelfde genetische element en dat gebeurt voornamelijk door integrons, transposons, IS elementen en fagen. Voor LGO zijn de drie genoverdracht systemen in bacteriën relevant: 1) conjugatie, 2) transductie en 3) transformatie en alle drie de systemen spelen een doorslaggevende rol bij overdracht van ARGs naar andere bacteriën. Laterale overdracht van ARGs via conjugatie gebeurt met behulp van plasmiden en/of ICEs, via transductie met behulp van fagen en via transformatie door cel opname van niet-celgebonden (extracellulair of ‘naakt’) chromosomaal, plasmide of faag DNA afkomstig uit de omgeving van de cel. Antibioticumselectiedruk speelt een sturende rol bij zowel rekrutering en klustering als bij LGO van ARGs. Selectiedruk vindt plaats in de kliniek en veehouderij, maar ook in landbouwsystemen en in natuurlijke omgevingen waarin bacteriën zich bevinden. ARGs worden in alle omgevingen aangetroffen, zelfs in omgevingen die nog niet zijn beïnvloed door menselijke activiteiten (pristine omgevingen) en in dierlijke resten ingevroren in permafrost en daterend uit de periode voorafgaand aan het antibioticum-tijdperk. In sommige gevallen zijn deze ARGs gekoppeld aan MGEs.

Rekrutering en clustering van ARGs zijn processen die in alle omgevingen onder selectiedruk zullen plaatsvinden en waarvan de tijdsduur langdurig is waardoor het onwaarschijnlijk is dat deze processen onder een kortstondige experimentele tijdsduur in het laboratorium (of andere ingeperkte omstandigheden) zullen plaatsvinden. Laterale overdracht van ARGs met behulp

van MGEs vindt ook plaats onder natuurlijke omstandigheden, maar deze processen zijn van korte tijdsduur en kunnen wel onder laboratoriumomstandigheden plaatsvinden. Voor het inschatten van risico's op het ontstaan van antibioticum resistente bacteriën is het belangrijk om te weten welke soorten ARGs aanwezig zijn in de gebruikte bacteriestammen en of deze genen daadwerkelijk gekoppeld zijn aan MGEs in het genoom, of niet. Afhankelijk van het type MGE kan er een inschatting worden gemaakt over de mogelijkheid tot overdracht van ARGs. In het geval van plasmiden kan de overdrachtsfrequentie hoog zijn, oplopend tot 10 – 50% van de ontvangende cel populatie. Bij sommige plasmiden is het gastheerbereik ook nog eens groot en deze typen van plasmiden (zoals IncP-1 type plasmiden die gekoppeld zijn aan colistine resistentiegenen) moeten worden beschouwd als 'worst case' met betrekking tot overdracht van ARGs. Risico's op overdracht van ARGs door andere MGEs, zoals fagen, transposons en IS elementen, worden lager ingeschat omdat het gastheerbereik smal is (in het geval van fagen), of omdat er eerst transpositie naar een andere MGE moet plaatsvinden voordat LGO daadwerkelijk kan plaatsvinden (in het geval van transposons en IS elementen). Op basis van de criteria gastheerbereik en overdrachtsfrequentie kunnen MGEs worden gerangschikt waarbij de kans op overdracht van ARGs op zelfoverdraagbare plasmiden het grootst is en op IS elementen en transposons het kleinst.

Summary

Antibiotic resistances in bacteria can be ‘intrinsic’, i.e. there is no target for the antibiotic substance present or the antibiotic molecule cannot reach the target site in the cell, or ‘acquired’, meaning that resistance is caused by genomic mutations in the cell or transmitted from other bacteria. Only acquired antibiotic resistance genes (ARGs) are relevant within the context of this report. Mobile genetic elements (MGEs) play key roles in the transmission of genes conferring resistance towards antibiotics. Eventual association of ARGs with MGEs in any type of bacterial species would indicate that ARGs were acquired via lateral gene transmission (LGT). The aim of the report was to indicate the most relevant aspects in relation to LGT of ARGs, especially the ones conferring resistances towards last resort antibiotics (LRAs) when associated with MGEs in bacterial genomes, and to define possibilities on lateral transmission in the laboratory.

The WHO list of critically important antimicrobials (https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/cia/en/) was used as a guideline for defining LRA. This group of antimicrobial compounds encompass third, fourth and fifth generations of cephalosporins and carbapenems, glycopeptides, macrolides and ketolides, polymyxins, quinolones, aminoglycosides, ansamycins, glycylcyclines and aminomethylcyclines, lipopeptides and oxazolidinones. Most attention in this report was on the most recently developed and approved agents within each antimicrobial class and, to a lesser extent, to the ‘older’ antimicrobials that already have been prescribed for over decades for medical and veterinary applications. A semi-systematic literature search (i.e. automatically searched in databases and then further selected out by hand on the basis of relevance within the framework of the report) in five library databases, was performed, based on the term ‘antibiotic resistance’ alone, or in combinations with ‘mobilome’, ‘resistome’, ‘microbiome’, ‘mobile genetic element’, ‘pangenome’, ‘gene’, ‘self-transmissible’ and/ or ‘host range’ as search terms. Selected references were then further selected out for the ones that report on ‘last resort antibiotics’. Based on these reports, it was concluded that many genes conferring resistances towards LRAs in both clinically relevant and environmental bacteria were associated with MGEs and thus were acquired via LGT from other bacteria. The diversity of these MGEs was high and included different types of integrons (located on transposons or plasmids), insertion (IS) elements, transposons, integrative conjugative elements (ICEs), plasmids and occasionally (pro) phages. Genes conferring resistances towards LRAs were

also found to be located on chromosomes, in the absence of MGEs, and chances on lateral transmission of these ARGs must considered to be negligible. MGEs are in most cases responsible for lateral transmission of ARGs.

Two steps are important to make ARGs mobile and transmissible and these are: 1) 'recruitment', i.e. gene rearrangement whereby ARGs are brought under alternative transcriptional control and this is mainly done by (super) integrons, and 2) 'clustering', i.e. placement of resistance genes on the same MGE, and this is mainly done by integrons, transposable elements and phages. For LGT, the three gene transmission mechanisms in bacteria are relevant: 1) conjugation, 2) transformation and 3) transduction and all three mechanisms play pivotal roles in lateral transmission of ARGs. Lateral transmission of ARGs towards other species via conjugation is done by plasmids and ICEs, via transduction by phages, and via transformation by uptake of non-cellular bound (extracellular or 'naked') chromosomal, plasmid or phage DNA present in the surroundings of the bacterial cell. Antibiotic selective pressure is the most important driver behind recruitment and clustering steps and LGO, and all these steps take place in clinical and veterinary environments, but also in agricultural production systems and in natural environments undisturbed by human activities. ARGs can be found in many different environments, including pristine ones, and ARGs were also found in animal bodies frozen in permafrost in the period predating the antibiotic era. In some cases these ARGs were associated with MGEs.

Recruitment and clustering of ARGs take place under selective pressure in all ecosystems and these are time-consuming processes, unlikely to take place in the short time frame realistic for experimentation in the laboratory (or in any other contained facility). Transmission of ARGs to other species is a relatively fast process that can occur in the short time frame, realistic for experimentation in the laboratory. For risk assessment purposes, it is important to know which type of ARGs is present in bacterial strains and whether they are associated with MGEs in the bacterial genome, or not. Assessment on the possibility for ARG transmission to other bacteria can be performed on the basis of the type of MGE present in the bacterial genome. In the case of plasmids, the transfer frequencies can be high to up to 10 – 50% of the recipient cell population. For some of these plasmids the host range can be broad (as in the case of the IncP-1 type of plasmid that was found to be associated with a colistin resistance, *mcr*, gene) and these plasmids must be considered as 'worst cases' with respect to lateral transmission of ARGs. Risk on transmission of ARGs with support of other MGEs, such as phages, transposons and IS elements, must be considered to be lower than of

plasmids, because of the narrow host ranges of these elements (in case of phages) or because of the fact that transposition to other MGEs is required as a first step, before LGT actually can take place (as is the case for transposons and IS elements). MGEs can be ranked on the basis of host range and transfer frequency as criteria, whereby the chance on ARG transmission will be highest when located on self-transmissible plasmids and lowest when located on, or closely associated with transposons and IS elements.

1 Motive, introduction and framework of the report

This literature study was commissioned by the *Commissie Genetische Modificatie,* (COGEM) to evaluate the aspects of presence of resistances to last resort antibiotics (LRAs), such as vancomycin, in bacterial strains that are used for genetic modification. Mobilization and transfer of genes encoding resistances towards LRAs to clinically relevant bacteria could jeopardize the antimicrobial treatment of patients with (serious) infectious diseases. For the COGEM it is important to gain a structured understanding on the possibilities and circumstances of lateral transmission of genes, encoding resistance to LRAs and present in bacterial strains, that are used for genetic modifications to other (pathogenic) micro-organisms. At this moment, pathogenicity of bacteria to humans, animals and plants is a relevant criterion in risk evaluation for experimentation with genetically modified micro-organisms under confined circumstances, such as in laboratory and large reactor settings. *Pathogenicity is an argument to upgrade the experimental containment level, under which the genetically modified micro-organisms will be applied, to the next higher containment level, and the question that will be addressed in this report is: which aspects play a role, from an environmental risk assessment perspective, in experimentation under confined conditions, such as in the laboratory with non-pathogenic bacteria that carry ARGs.* Pathogenicity is, however, a term that is hard to define because it also depends on the health status of the patient. Therefore, for bacterial species in clinical contexts the term 'clinically relevant bacteria' will be further used in this document.

LRAs do not constitute a well-defined class of antibiotics. The WHO classified antibiotics to be 'medically important antimicrobials' (https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/cia/en/) (last updated version was in 2016) on the basis of two criteria: 1) possibility for alternative antimicrobial strategy, in case of allergy, toxicity or resistance, and 2) antimicrobial therapy in case of life-threatening infection. The different classes of medically important antimicrobials were further ranked in importance (Anonymus, 2017, WHO list of critically important antimicrobials) and the group of critically important antimicrobials, both of high and of highest priority categories, was used as guideline for this report. Thus, the focus will be on the most recently developed and approved agents within each antimicrobial class (Table 1), and less attention will be paid to the 'older' antimicrobials that are already prescribed for over longer periods in time for medical and veterinary use. Not all antimicrobial classes will fit within the context of

this report, such as the classes of monobactams, penicillins, phosphonic acids and drugs that are only used for the treatment of mycobacterial diseases, including tuberculosis.

ARGs in clinically relevant bacteria can be acquired and mobile genetic elements (MGEs) play key roles in the transmission of these genes. Associations between ARGs and MGEs occur, however, both in clinically relevant as well as in environmental bacteria and therefore the term ‘bacteria’ refers to both groups in this document, unless specifically mentioned. Eventual association of genes conferring resistance towards LRAs with MGEs in bacteria would imply higher chances on lateral transmission of this group of ARGs to clinically relevant bacteria. Association of ARGs with MGEs can be defined as ARGs that are physically located on, or located at a proximate distance from MGEs in the bacterial genome. Considering this criterion, a list of genes conferring resistances towards LRAs was made by making use of a semi-systematic literature search (i.e. automatically searched in databases and then further selected out by hand on the basis of relevance within the framework of the report) (Supplement Fig. S1). The main focus will be on later generations of existing, and on recently approved antibiotics among the different classes of LRAs. Based on most frequently observed associations between MGEs and ARGs, a generic model was made describing the different steps in clustering and mobilization of ARGs. By making use of this model, individual cases can be assessed by weighing the chances on lateral gene transmission (LGT) to other bacteria, especially to clinically relevant bacteria, on the basis of presence or absence of associations with different types of MGEs. For that purpose different LGT types were defined for experimentation with bacteria carrying ARGs associated with MGEs under contained (laboratory) circumstances, based on LGT mechanisms (conjugation, transformation and transduction). Different criteria were therefore set with respect to lateral transmission properties of the genetic element, such as possibilities for self-transmission, transfer frequency and bacterial host range.

Table 1. Bacterial targets and resistance mechanisms of different LRA classes

Antibiotic class	**Bacterial targets**	**Resistance mechanism**
Cephalosporins and Carbapenems	Gram negative bacteria (Proteobacteria)	Inhibition of cell wall synthesis
Glycopeptides	Gram positive cocci	Inhibition of cell wall synthesis
Macrolides and ketolides	Gram positive bacteria (some Gram negative)	Inhibition of protein synthesis
Polymyxins	Gram negative bacteria (Proteobacteria)	Cell membrane damage
(Fluoro) quinolones	Gram positive and Gram negative bacteria	Inhibition of DNA replication and transcription
Aminoglycosides	Gram negative bacteria (Proteobacteria)	Inhibition of protein synthesis
Ansamycins	Gram negative and some Gram positive bacteria	Inhibition of DNA transcription
Glycylcyclines	Gram positive and Gram negative bacteria	Inhibition of protein synthesis
Aminomethylcycline	Gram positive and some Gram negative bacteria	Inhibition of protein synthesis
Lipopeptides	Gram positive bacteria	Cell membrane depolarization
Oxazolidinones	Gram positive bacteria	Inhibition of protein synthesis

2 History of antibiotics and emergence of bacteria resistant to antibiotics

The increase of antibiotic resistance in bacterial pathogens is of major concern for the antimicrobial treatments involving antibiotics in the combat against infectious diseases in humans and companion animals. Since the discovery of penicillin by Alexander Fleming in 1928, antibiotics are widely used in medical treatments, but later also in food production by using antibiotics as livestock feed supplements to improve overall animal health and production yields (Ventola, 2015). The first large scale use of antibiotics (penicillin) occurred on allied soldiers in 1943 for the treatment of wound infections, mostly caused by *Staphylococcus* species. In about the same time period the first resistance against penicillin was already reported. However, new antibiotics soon arrived; tetracycline in 1950, erythromycin in 1953, methicillin in 1960, gentamicin in 1967 and vancomycin in 1972 (Ventola, 2015).

However, soon after introduction of new antibiotics, resistances against these agents also appeared. First was tetracycline resistance observed in *Shigella* species (1959), later followed by methicillin resistance in staphylococci (1961), erythromycin resistance in *Streptococcus* species (1968) and gentamicin resistance in enterococci and coagulase-negative staphylococci (1979). Next generation antibiotics were developed to extend bacterial activity spectra or to cure infections caused by bacteria that possessed resistances against the 'older' antibiotics. For example, penicillins were replaced by cephalosporins and carbapenems to treat infectious diseases caused by pathogens producing enzymes (β-lactamases) inactivating the 'older' β-lactam antibiotics, or these older antibiotics were combined with newly developed β-lactamase inhibitors (e.g. clavulanic acid) in medical treatments. Beta-lactamase inhibitor avibactam could protect antibiotic activities of ceftazidime, ceftaroline and aztreonam against different classes of β-lactamases (Giani et al., 2016) and therefore these agents are still of medical importance.

Again resistances to later generations of antibiotics appeared and also accumulation of (extensive) resistances against different groups of antibiotics (multiple resistances) or even to all clinically available antibiotics (pan-resistance) were reported in single bacterial lineages, especially in non-fermenting bacteria such as the ones belonging to *Pseudomonas* and *Acinetobacter* species (Miriagou et al., 2005). The 'arms race' against antibiotic resistances resulted in recovery and (semi) synthetic design of the latest generations of antibiotics that are

under approval, or that were recently approved. Dalbavancin, oritavancin, and telavancin (all three semisynthetic lipoglycopeptides), cefiderocol (cephalosporin), colistin (polymyxin), daptomycin (lipopeptide), eravacycline and omadacycline (both aminomethylcyclines), fidaxomicin (macrocyclic antibiotic), finafloxacin (fluoroquinolone), 8-hydroxyquinoline derivatives, plazomicin (aminoglycoside), tedizolid (oxazolidone) and tigecycline (glycylcycline) are the last new-coming antibiotic compounds that appeared on the market (Macone et al., 2014, Chaudhary, 2016; Mazer-Amirshahi et al., 2017; Lawung et al., 2018).

Worrying are the high and still increasing percentages reported on antibiotic resistances in Gram-negative bacteria, especially resistances against third-generation cephalosporins, often in combination with resistances against fluoroquinolones and aminoglycosides (Chaudhary, 2016). For Gram-positive bacteria, the increase in resistance against methicillin in *Staphylococcus aureus* (so called methicillin-resistant *S. aureus* [MRSA]) and vancomycin in enterococci is worrying (Chaudhary, 2016). Among a survey over 19 hospitals in the US from 2007 – 2010, percentages vancomycin resistance in *Enterococcus faecium* among 4024 isolates was 87.1%, oxacillin/methicillin resistance in *S. aureus* was 56.8% (more than 23,477 isolates), clindamycin resistance in *S. aureus* was 39.7% (21,133 isolates), fluoroquinolones resistance in *Pseudomonas aeruginosa* was 32.6% (10,982 isolates), fluoroquinolones resistance in *Escherichia coli* was 31.3% (30,715 isolates), and daptomycin resistance in *E. faecium* was 3.9% (2,029 isolates) (Edelsberg et al., 2014). Currently, the status of infectious diseases must be revisited and worldwide reported diseases caused by the ESKAPEE group of pathogens (*E. faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa, Enterobacter* spp. and *E. coli* [Partridge et al., 2018]) has become threatening again due to the high antibiotic (multi) resistance levels among this group of pathogens.

The use of antibiotics is still rising in low and middle income countries, particularly, the use of the so-called LRAs such as carbapenems, glycylcyclines, oxazolidinones and polymyxins (Klein et al., 2018). Global increase in the use of antibiotics, and especially misuse and use in veterinary and agricultural production systems are considered to be the main drivers behind increased antibiotic resistances in different groups of bacteria (Stokes and Gillings, 2011; Ventola, 2015, Klein et al., 2018; Harmon et al., 2018). However, no epidemiological linkage was found in ESBL/*amp*C genes and plasmids in microbial populations from livestock farms and from humans (Dorado-Garcia et al., 2018; Ceccarelli et al., 2019). LRAs, such as carbapenems, are reserved for clinical treatments only, whereas

other β-lactam antibiotics (third and fourth generation cephalosporins, such as ceftiofur, cefquinome and cefoperazone), aminoglycosides, colistin and fluoroquinolones (enrofloxacin, danofloxacin, difloxacin and marbofloxacin), that are used for antimicrobial treatment of humans, can only be used in animals under restricted circumstances in the Netherlands (Anonymous, SDa autoriteit Diergeneesmiddelen, 2016). Persistence of antibiotic residues in animal faeces can be long, depending on the chemical composition of the antibiotic compound (Kyselková et al., 2013; Berendsen et al., 2018). Based on frequency of use and persistence, oxytetracycline, doxycycline, flumequine and tilmicosin antibiotics were expected to end up in the environment as result of manure applications to arable land. Accumulation and persistence of antibiotics in natural ecosystems will lead to selective pressure on environmental bacteria resulting in increased resistances in different groups of bacteria.

Applications for recently approved antibiotics are strictly regulated to prevent accumulation of antibiotic resistances in pathogens. LRAs cover a diverse group of antimicrobials. This list includes carbapenems (ertapenem, doripenem, imepenem, meropenem), but also drugs that are new on the market such as tigecycline, colistin and the other drugs mentioned before. In fact colistin is an 'older' antibiotic (polymyxin E) that reappeared on the market as a solution for pathogens that are already resistant to other antibiotics (McKenna, 2013; Osei Sekyere, 2016). Glycopeptides, first generation vancomycin and teicoplanin, and second generation (semisynthetic) dalbavancin, oritavancin and telavancin, also are considered as LRAs (Binda et al., 2014; Marcone et al., 2018). Daptomycin is a more recently approved antibiotic that is used for treatment against MRSA infections, but also for patients who developed allergies for β-lactams (Sabat et al., 2018). Resistances against daptomycin are reported, but still rare (Palmer et al., 2011; Bayer et al., 2013; Sabat et al., 2018). Linezolid was approved by the US Food and Drug administration (FDA) in the year 2000 and resistances against this drug are reported for staphylococci and enterococci and therefore its use is only approved for antimicrobial treatment of humans only, and not for animals (Endimiani et al., 2011, Gu et al., 2012, Kuroda et al., 2018, Tyson et al., 2018). The introduction of β-lactamase inhibitors in new medicine combinations restored antibiotic activities of older generations of cephalosporins and therefore these drugs regained their status as LRA.

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3 Mobile genetic elements associated with antibiotic resistance genes

3.1 Association of ARGs with MGEs

A list of genes conferring resistances towards LRAs was created based on information from literature. Eventual association of these ARGs with MGEs is summarized in Table 2. Based on this information, it can be concluded that mobility of ARGs towards (last resort) antibiotics commonly is associated with MGEs. Lateral transmission of these ARGs resulted in selection for specific strains or even clonal lineages within the same species. The diversity of MGEs associated with ARGs is high and ARGs against LRAs were associated with all existing types of MGEs, including integrons (only mobile when located on plasmids or transposons), insertion elements (IS), transposons, integrative conjugative elements (ICEs), plasmids and occasionally (pro) phages. ARGs were also found to be located on chromosomes, in the absence of MGEs, within the same strain or clonal lineage, but these genes must be regarded to be less mobile in comparison with the ones associated with MGEs. The role of these different MGE types in lateral transmission of ARGs will be discussed into more details in the next subsections.

3.2 Transfer of antibiotic resistances.

Two types of genetic transmission routes exist for (antibiotic resistance) genes among prokaryotes; vertical and lateral (synonymous for horizontal) gene transmission (Stokes and Gillings, 2011).

Vertical transmission. In vertical gene transmission, genes are transmitted to the next generations of cells via DNA replication and cell division. Vertically transmitted ARGs are thus spread clonally to succeeding generations. For transmission of ARGs, this type of transmission seems to be relevant because it is often the case that variants of ARGs are associated with particular sequence types (STs) and clonal lineages as shown for *E. coli* (Chen et al., 2016; Luo et al., 2018) and *K. pneumoniae* (Yong et al., 2009; Casella et al., 2018). The toxin-antitoxin gene systems located on plasmids secure vertical transmission of plasmids via clonal offspring, because offspring cells lacking the plasmid will be killed by the toxin because of degradation of the unstable antitoxin. However, the genetic background of the cell line carrying the ARG also will play an important role in the ecological success of the

clonal line. For example, in *E. faecalis* it was shown that one particular strain carrying the linezolid resistance gene *cfr* also carried multiple adhesion properties on its chromosome that were presumed to be involved in biofilm formation (Kuroda et al., 2018). The combination of chromosomally-encoded biofilm formation genes and transposon Tn*6218*-borne linezolid resistance provides ecological competence properties to this clonal lineage. Two clonal *E. coli* lineages, denoted as A-ST167 and A-ST410 and originating from, respectively, beef and turkey meat samples, both produced CTX-M-15 β-lactamase and there was a clear link between the gene encoding CTX-M-15 β-lactamase and IncF plasmids (Irrgang et al., 2017). Because both lines were shown to be prevalent among humans, it was assumed that they were transmitted to humans via food consumption. The combination of antibiotic resistance and ecological properties to survive on meat and to colonize the human intestinal track made both lineages ecologically competent under these specific and selective circumstances.

Lateral transmission. In lateral transmission events, genes are transferred to cells of the same and of other species, thus potentially crossing taxonomic borders even up to the kingdom level (Frost et al., 2005). The definition for LGT within the scope of this report is taken over from Stokes and Gilling (2011) that defines LGT as ".... the process whereby the DNA from one cell is physically transferred from one cell to another without an absolute requirement for cell division and the incorporation of that DNA into the recipient's genome such that it can be stably inherited". For LGT, the three canonical processes leading to genetic spread across (bacterial) species borders are important and these are: 1) conjugation, 2) transformation and 3) transduction (Frost et al., 2005).

For conjugation, physical contact between donor and recipient cells is required and DNA is transferred via conjugation pili that connect donor with recipient cells during the DNA transmission process. DNA of conjugative genetic elements is transferred via these so-called sex pili that are assembled from proteins encoded from type IV secretion systems. Type IV secretion systems are highly conserved DNA signatures in conjugative elements in Gram-negative bacteria (Frost et al., 2005). Distinction between conjugative plasmids can be made between the ones that are self-transmissible and those that are not. This last group of conjugative non-self-transmissible, so called mobilisable, plasmids rely on the presence of other (self-transmissible) plasmids in the same bacterial cell for conjugation. Many different types of MGEs, such as plasmids and ICEs, involved in conjugation are associated with ARGs (Table 2), indicating that conjugation plays an important role in lateral transmission of ARGs among bacteria.

Transformation is defined as the uptake of extracellular DNA by the cell, in the form of 'naked' DNA (i.e. unprotected by a cell wall or membrane), from its local environment. Therefore DNA uptake, integration into the host cell genome and expression are crucial steps in the transformation process. For integration of (intact) plasmid or phage DNA into the host bacterial genome, no homology between incoming and host DNA is needed. However for integration of chromosomal DNA into the host genome, extensive DNA similarity between new incoming and host genome DNA is needed. Homologous recombination leads to incorporation of foreign DNA into the host genome and this process is mediated by proteins encoded on the host chromosome and occasionally homologues of these genes are located on bacteriophage genomes. For transformation, competence of the receiving bacterial cell for DNA uptake is needed and nowadays competence has been described in 60 species including clinically relevant species such as *S. pneumoniae* and *Acinetobacter baylyi* (Domingues et al., 2012). Like for conjugation, transformation can also lead to drastic changes in the antibiotic resistance profiles of the receiving strains (Domingues et al., 2012).

Transduction is transmission of foreign DNA from donor to recipient cells via bacteriophages. Transduction mostly occurs by 'wrong' excision of prophage DNA from the donor chromosome, allowing incorporation of phage DNA with additional chromosomal DNA fragments, into the phage capsid. There are two types of transduction, generalized and specific transduction. In generalized transduction, any type of bacterial DNA is incorporated into the phage capsid, whereas in specialized transduction a specific set of genes flanking the phage incorporation side on the bacterial chromosome is incorporated into the phage capsid. Generalized transduction is a relative rare event whereas specialized transduction occurs more often, for example in *E. coli* by phage λ. ARGs were found to be present in bacteriophage DNA pools from environmental samples, including β-lactamase Ambler class D, glycopeptide and polymyxin resistance genes (Subirats et al., 2016) and the colistin resistance *mcr*-1 gene (Wang et al., 2018a). Relative abundances of ARGs in bacteriophage DNA pools were between < 0.001 and 0.26% in the studies of Subirats et al. (2016) and Wang et al. (2018a). Therefore, bacteriophages present in microbial communities, the so-called virome, must be considered as potential sources of ARGs, including the ones classified in this report as LRAs Lekunberri et al., (2017). However, the host range of the vast majority of phages is limited to strains within the same species.

3.3 ARG capturing, gene shuffling and formation of gene cassettes

ARGs evolved from ancestor genes that not necessarily played roles in antibiotic resistances. However, at the start of the antibiotic era the modern forms of ARGs evolved from these 'ancestor ARGs' by subsequent mutation events (Garmendia et al., 2012; Perry and Wright 2013). Most likely ancestral genes of ARGs are millions of years old and some are supposed to play important roles in the defence of antibiotic producers in soil habitats as was the case for soil-dwelling and vancomycin producing *Amycolatopsis orientalis* (*Actinobacteria*) strains (Binda et al., 2014). These ancestral ARGs are presumed to still exist in areas that are undisturbed by human activities, such as in bogland in the Austrian Alps (Obermeier, TU Graz, unpublished results). The organization of genes involved in antibiotic resistance in clinically important bacteria is, however, often different from the ones found in the original antibiotic-producing bacteria as for example shown in the *van*A operon located on transposon Tn*1546* (Kohler et al., 2018) (Table 2).

Gene capture and shuffling of open reading frames into small discrete mobile elements often containing integrons, so called gene cassettes, result in reshuffling of ARGs thereby bringing ARGs under transcriptional control of strong promoters. Integrons play pivotal roles in chromosomal gene arrangement processes in different bacterial phyla and therefore are important in the rapid adaptation of bacteria to environmental changes (Rowe-Magnus and Mazel, 2002). Integrons are ancient structures in bacteria, predating the antibiotic era and evolved from ancestral structures named 'super-integrons'. Most likely super-integrons act as substrates in the formation of the more modern forms of integrons by capturing resistance loci. This was followed by integrating them into mobile and high copy number plasmids (Rowe-Magnus and Mazel, 2002). Thereby, promoterless open reading frames are circularised and integrated, with the help of an integron (*int*I)-encoded integrase, into specific attachment (*att*C) sites located elsewhere on the genome and this can be the bacterial chromosome, but can also be other genetic elements such as other integrons, transposons, plasmids or (pro) phages (Gillings et al., 2015). Open reading frames located on these circularised DNA fragments are placed in an operon under the transcriptional control of two promotors (Pc or P1 and P2) located on the integron (Rowe-Magnus and Mazel, 2002; van Hoek et al., 2011; Partridge et al., 2018). Sometimes, open reading frames are integrated upstream of (strong) promoter regions in integrons and these genes will not be expressed. Bacterial strains carrying non-transcribed open reading frames will be identified as antibiotic sensitive upon phenotypic screening for antibiotic resistance, whereas the structural part of the gene still can be present

in the bacterial genome (Rowe-Magnus and Mazel, 2002; Partridge et al., 2018). This might be of relevance in case genomic rearrangements in bacterial cells lead to reshuffling of these non-transcribed regions, bringing them under the control of alternative, host-controlled, promoter regions.

Integrons thus did not evolve during the antibiotic era, but selective pressure conferred by medical and veterinary use of antibiotics accelerated the evolution of integron formation carrying (multiple) antibiotic resistance cassettes (Gillings et al., 2015). All genes are co-expressed in multi-resistance integrons leading to co-selection for antibiotic resistances or to genes conferring resistances towards heavy metals and disinfecting agents (quaternary ammonium compounds) (Gillings et al., 2015). Nowadays at least five classes of integrons are defined of which some are linked to transposable elements, such as integron class 1 to Tn*21* and integron class 2 to Tn*7* (Rowe-Magnus and Mazel, 2002; Stokes and Gillings, 2011; Partridge et al., 2018).

3.4 Transposable elements and plasmids

Many different families of insertional (IS) and transposable elements are nowadays identified and described in literature and often these genetic elements are specific for different groups of Gram-positive and Gram-negative bacteria (Partridge et al., 2018). However, in general, transposable elements can be distinguished into the ones that can only integrate into new locations in the bacterial genome, or that carry all required genes for conjugative transfer in their origin of transfer regions (*ori*T), the so called ICEs (sometimes also named conjugative transposons) (Hall et al., 2017; Partridge et al., 2018). Transposable elements such as IS elements and transposons are discrete DNA fragments that duplicate, or that excise from old, and integrate into new locations in the bacterial genome and these new locations can be the bacterial chromosome, or any other MGE present in the bacterial cell such as plasmids or other transposable elements (Zhang et al., 2017; Partridge et al., 2018).

Plasmids are autonomously replicating DNA fragments in bacterial cells and most often these fragments are circular, but sometimes linear. Plasmids consist of so called back bone (core) genes, e.g. housekeeping genes that are responsible for maintenance in the cell (e.g replication, toxin-antitoxin production and copy number control), and accessory genes, i.e. genes that are not necessarily important for maintenance in the bacterial cell, but rather confer additional ecological fitness to the host, such as ARGs (van Hoek et al., 2011).

Accessory genes are acquired via genetic transfer and recombination events. Different groups of plasmids are described (van Hoek et al., 2011; Partridge et al, 2018) based on structural differences in backbone genes. In general, plasmids are distinguished on the basis of their compatibility with other plasmids in the same bacterial host. Plasmids incompatible in the same host belong to the same incompatibility (Inc) group and thus far 26 different compatibility groups have been described for Enterobacteriaceae, 14 for Pseudomonads and 18 for Gram-positive, mainly enterococci and staphylococci, species (Frost et al., 2005). Molecular sizes (mainly determined by their accessory gene load), transmission frequencies and recipient host ranges are typical features for plasmids, and plasmids substantially can differ from each other, even within the same Inc group.

As mentioned before, plasmids can be distinguished into the groups of conjugative and non-conjugative plasmids; i.e. plasmids that can be transferred to other bacteria via conjugation, carrying their own origin of transfer (*oriT*), and those that cannot. Among the group of conjugative plasmids there are the plasmids that are self-transmissible; i.e. containing the complete set of genes that is required for transmission to other bacteria, known as *tra* genes, and those that lack these *tra* genes and that are unable to transfer themselves to other bacteria, but that can rely on transfer functions provided from other plasmids *in trans*. Conjugative plasmids that are transmitted with the help of self-transmissible plasmids are mobilizable plasmids. For transfer of mobilizable plasmids, three parent cells are needed, the recipient and two donor cells separately hosting a mobilizable or a self-transmissible plasmid. The IncQ type of plasmids belong to the group of mobilizable plasmids and the host range of this group of plasmids is extensive conferring large promiscuity to these type of plasmids. IncP (especially InP-1) and PromA (Heuer and Smalla, 2012) type of plasmids are examples of self-transmissible plasmids and these plasmids were found in soil environments where they showed the remarkable capacity to mobilize or retromobilize IncQ plasmids (Heuer et al., 2002 and van Overbeek et al., 2002). Plasmids can further be distinguished in host range sizes and IncF, IncH and IncI type of plasmids are generally regarded as narrow host range, whereas IncN, IncP and IncW as broad host range type of plasmids (Suzuki et al., 2010). Conjugative plasmids and ICEs are the most important vehicles for lateral transmission of ARGs across the bacterial phylogeny spectrum, occasionally crossing kingdom barriers. The main difference between ICEs and conjugative plasmids is that conjugative plasmids carry all required genes for autonomous replication in the bacterial cells (origin of replication), whereas ICEs solely depend on chromosomal replication (van Hoek et al., 2011; Hall et al., 2017; Partridge et al., 2018).

Many different types of plasmids can be present within closely related bacterial strains as was the case for the *E. coli* ST131 lineage. This lineage revealed the presence of 39 different plasmids classified on the basis of different properties such as Inc type and on protein sequences translated from plasmid backbone genes (Lanza et al., 2014). Of these 39 plasmids, 11 were identified as IncF2 plasmids, whereas the others were identified as IncN, I1/K/BO, I2, A/C and X. Additionally, many cryptic plasmids were found that could not be further identified because the open reading frames on these plasmids had not been annotated yet. Within the largest group of identified plasmids, the IncF2 plasmids, carbapenem resistance genes bla_{KPC} and bla_{NDM} were present. The presence of such a wide diversity of plasmids within the same single lineage of *E. coli*, the so called plasmidome, indicates that many different plasmids may occur in the same bacterial strain including hitherto unidentified plasmids. This is an important message within the context of the report because it indicates that lateral transmission of ARGs associated with MGEs in bacterial strains will remain unpredictable, especially when genomic (plasmidome) data are not available. For example, the clustering of bla_{NDM-5} and *mcr*-1 genes, conferring resistances to, respectively, carbapenems and colistin, were both located on the same hybrid IncX3-X4 plasmid in *E. coli*, that most likely resulted from fusion of two separate plasmids that co-occurred in the same cell (Sun et al., 2016). Co-location on the same single plasmid lead in co-transfer of antibiotic resistance loci, with transfer frequencies of between $4 – 7 \times 10^{-5}$ per recipient cell. With these frequencies, lateral transfer of clustered carbapenem/colistin resistances are realistic in infections of humans, where bacterial cell densities in pus can reach numbers of 10^8 per ml or higher.

3.5 *The role of biofilms in lateral gene transfer of antibiotic resistances and recovery of antibiotic resistances from uncultured bacteria.*

LGT via conjugation, transduction and transformation takes places in all microbial environments. However, the circumstances under which these gene transfer events take place may differ for each of the three gene transfer mechanisms. For a long time, bacterial attachment to solid phases was considered to be prerequisite for conjugation, although the concept of conjugation also taking place between bacterial cells living in planktonic stages became more recently accepted (Frost et al., 2005; Lim et al., 2017). However, LGT is commonly associated with the formation of (mixed) microbial biofilms in diverse ecosystems (Hannan et al., 2010; van Overbeek and Saikkonen, 2016; Lim et al., 2017).

Early biofilm formation of *E. faecium* led to upregulation of 177 genes of which many were involved in bacterial cell adherence, plasmid replication and LGT (Lim et al., 2017). Also, expression of *tet*C was upregulated leading to enhanced ribosomal protection against tetracyclines which appeared to be relevant in biofilm formation because expression of the ribosomal genes was downregulated at the initial stage of biofilm formation. This observation coincides with the general observation of high resistance of bacterial cells present in (mixed) biofilms towards deleterious agents, such as antibiotics. However, antibiotic resistance is also subscribed to the presence of inactive cell forms in biofilms as many bacterial cells in biofilms are in a metabolically inactive state. The co-occurrence of chromosomally-encoded biofilm formation genes and a transposon Tn*6218*-borne linezolid resistance in *E. faecalis* isolate KUB3006 (Kuroda et al., 2018) are suggestive for the importance of biofilm formation for lateral transfer of ARGs. In a mixed oral microbial community growing as biofilm, the transmission of transposon Tn*916* loaded with a tetracycline resistance gene was observed between *Veillonella dispar*, acting as donor, to *Streptococcus* spp., acting as recipients (Hannan et al., 2010). Many bacteria in natural ecosystems live as microbial consortia in biofilms (Flemming and Wingender, 2010) and the role of biofilms in lateral gene transmission via broad-host range plasmids is a rather unexplored phenomena in science to date (Klümper et al., 2014).

Exploration of bacterial genes in general (metagenomics), or of ARGs (resistomics) or MGEs (mobilomics) in specific, among uncultured bacteria via next generation sequencing is an upcoming field in microbiological sciences (Frost et al., 2005). Different classes of β-lactamase genes were found in human gut microbiomes, even from individuals that never had been exposed to antibiotics in their lives before (Garmendia et al., 2012). There are thus strong indications that resistances towards clinically relevant antibiotics, including some that are classified as LRA such as vancomycin, are linked to environmental resistomes. This altogether makes clear that ARGs commonly are present in all natural environments (Heuer et al., 2002; van Overbeek et al., 2002) and that occasionally environmental habitats are the sources of ARGs that are commonly found in clinically relevant bacteria. Resistome analyses on pan genomes of clinically important pathogens may become a new diagnostic tool for the recovery of genes involved in resistances against recently approved antibiotics (Kruse et al., 2014; Liu et al., 2016; Sabat et al., 2018).

3.6 A generic model for lateral transfer of ARGs and the role of selective pressure

ARGs originate from natural environments (soil) where they protect antibiotic producers from their own toxic products. A three-step generic model provides insight in mobilization and lateral transmission of ARGs under (antibiotic) selective pressure. The first step is recruitment of ARGs which is mainly done by (super) integrons as shown before. The second step is clustering of all genes required for cellular protection against antibiotics, inactivation of antibiotics or for pumping antibiotics out of bacterial cells, but also for clustering of multiple antibiotic resistance pathways into resistance islands and this is mainly done by integrons, transposable elements and phages. The third step is the transmission of ARGs or resistance islands towards other species and that can occur via conjugative mobile elements (conjugation), via DNA from lysed cells (transformation) or via bacteriophages (transduction). These steps are presented in Fig. 1. ARGs in environmental and clinically relevant strains that are acquired via LGT are always associated with MGEs. For example, in *Desulfitobacterium hafniense* (Kruse et al., 2014), the clustering of all genes responsible for vancomycin resistance (step 2 in Fig 1) appeared to be incomplete. The *van*H homologue was located outside the *van*I operon, making lateral transmission of the complete and functional operon into an unlikely event.

Important is the role of selective pressure in recruitment, clustering and lateral transmission of ARGs (Fig. 1). As shown before, selective pressure is the most important driver behind all steps involved in lateral transmission of ARGs. Other factors influencing LGT are factors related to plasmid type such as, self-transmissibility, transfer rate and host range, but also environmental factors such as local nutrient status (high nutrient availability stimulate microbial activities including LGT), cell density, attachment to solid surfaces and/ or presence of biofilms. Selective pressure takes place in clinical and veterinary environments where antibiotics are used, but can also take place in agricultural and natural settings, e.g. upon manure applications (Kyselková et al., 2013; Berendsen et al., 2018) and wastewater discharges (Adamczuk and Dziewit, 2017; Khan et al., 2018). Relevant in this aspect is the co-selection for ARGs when multiple resistance genes are clustered into genomic islands. Selective pressure by a single antibiotic, heavy metal or disinfecting agent can already lead to co-selection and lateral transmission of many other resistances, including the ones that are classified as LRAs, as was the case for colistin and carbapenem resistance genes that were co-located on the same (hybrid) plasmid (Sun et al., 2016). Relevant in this aspect is also that open reading frames of ARGs in the recruitment and/or clustering steps are not always

brought under transcriptional control of a (strong) promoter as shown before. Unexpressed ARGs in bacterial genomes will be missed by phenotypic selection, but can become controlled by an alternative promoter in later offspring upon genomic rearrangements, e.g. via transpositions.

Fig. 1. Schematic presentation in the order of events of recruitment, clustering and lateral transmission of ARGs in clinical and non-clinical environments under antibiotic selective pressure.

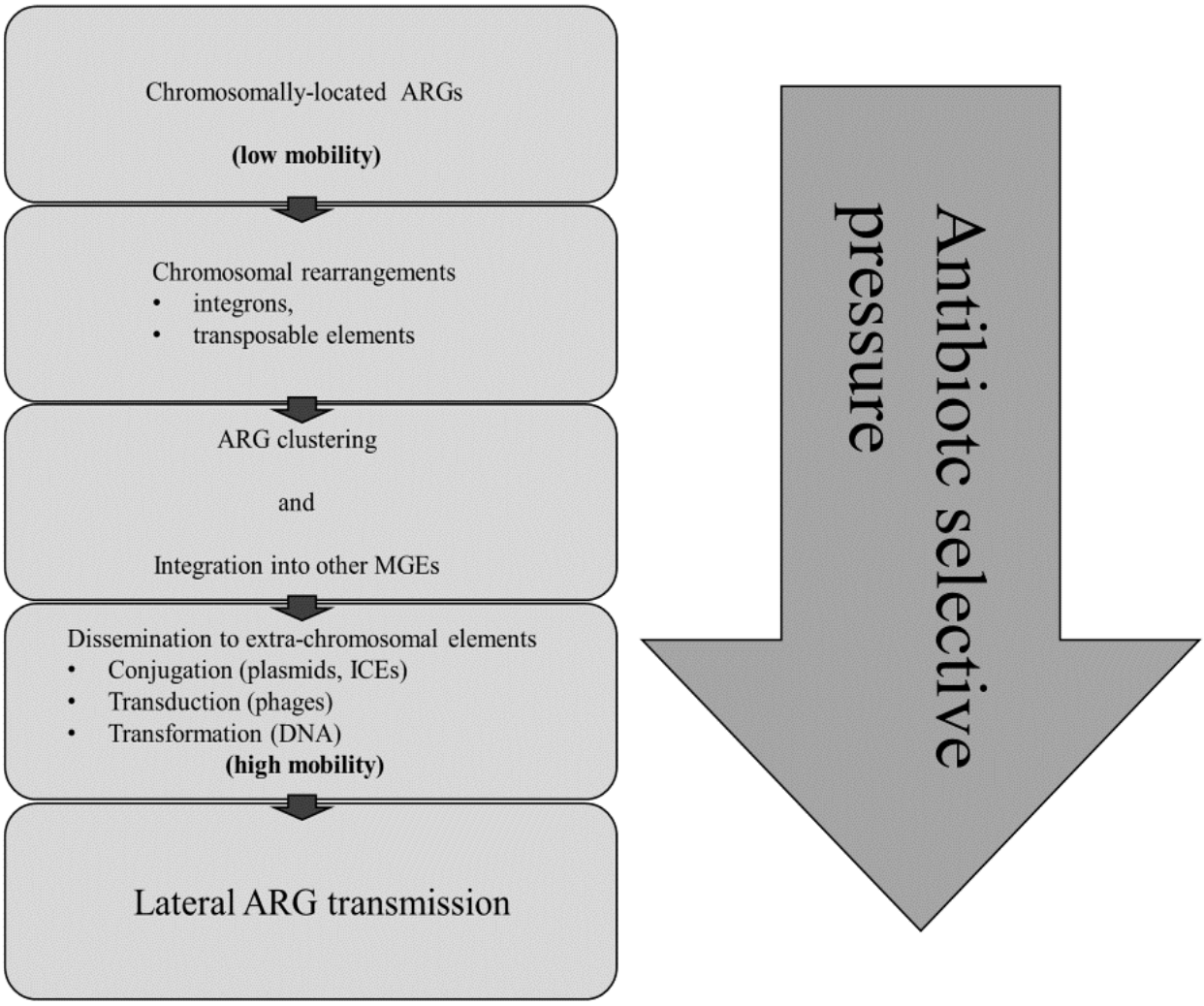

Table 2. Antibiotic resistance classes and antibiotic resistance genes/ operons associated with MGEs prioritized in accordance with the WHO list of critically important antimicrobials (2016 version available on internet: https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/cia/en/).

Antimicrobial class (antibiotics) and ARGs/ operons*	**Associated MGEs**	**References**
Cephalosporins and Carbapenems (meropenem, imepenem, ertapenem, doripenem)		
bla$_{KPC}$	ISCR2, ISCR3, IS*26*, Tn*3*, Tn*1721*, Tn*4401*,Tn*5393* and IncFII, FIA, FIB, I2, A/C, N, X, R, P, U, W, W, L/M and ColE plasmids	Yigit et al (2003), Mathers et al., (2011), Chen et al (2014); Bi et al (2015), Cerdeira et al. (2017), Luo et al (2018), Casella et al (2018)
bla$_{IMP}$	class 1 and 3 integrons, Tn*21*, Tn*5051*, and Inc A/C, IncHI2, Inc L/M, IncU plasmids	Poirel and Nordmann (2006), Caratolli (2009), Walsh (2010), Potter et al (2016), Luo et al (2018), Ahmad et al. (2018)
bla$_{NDM}$	Class 1 integron, IS*26*, Tn*3*, Tn*125*, Tn*300*, and IncF, IncFII, IncR, IncX3 and undetermined plasmids	Yong et al. (2009), Miriagou et al. (2010), Walsh (2010), Bush (2013), Potter et al. (2016), Luo et al (2018)
bla$_{VIM}$	Class 1 (complex) integrons, Tn*402*, Tn*5090* and Inc A/C, IncFI/II, Inc HI2, IncI1, Inc L/M and IncN, IncW plasmids	Miriagou et al. (2005), Caratolli (2009), Walsh (2010), Potter et al. (2016), Ahmad et al. (2018)
bla$_{OXA}$	IS*1999*, IS*Aba1*, IS*Aba2*, IS*Aba3*,Tn*2006*,	Poirel and Nordmann (2006), Walther-Rasmussen and Høiby (2006), Miriagou et al (2010), Walsh (2010),
Glycopeptides (vancomycin, teicoplanin, televancin, dalbavancin, itavancin)		
*van*A*	Tn*1546*, IncI8 plasmids	Hegstad et al., (2010), Binda et al. (2014), Kohler et al., 2018, Marcone et al., (2018), Partridge et al., 2018),
*van*B*	Tn*1549*, Tn*5382*-like conjugative transposons (ICEs), pCF10-like plasmid	Hegstad et al., (2010), Binda et al. (2014), Marcone et al., (2018), Partridge et al., 2018)
*van*C*	chromosome	Hegstad et al., (2010), Binda et al. (2018)
*van*D*	chromosome	Hegstad et al., (2010), Binda et al. (2018)
*van*E*	chromosome	Hegstad et al., (2010), Binda et al. (2018)
*van*G*	chromosome/ ICE	Hegstad et al., (2010), Binda et al. (2018)
*van*I*	chromosome	Kruse et al. (2014)
*van*L*	chromosome	Hegstad et al., (2010), Binda et al. (2018)
*van*M*	chromosome/ plasmid	Binda et al. (2018)
*van*N*	plasmid	Hegstad et al. (2010), Binda et al. (2018)

Antimicrobial class (antibiotics) and ARGs/ operons	Associated MGEs	References
Macrolides and ketolides (erythromycin, clarithromycin, azithromycin, fidaxomycin)		
erm A,B, C, T, Y	genomic islands, transposons, Tn*5253*-like composite ICE, plasmids, phage (ΦJN4341-pro)	Chaffanel et al. (2015), Chancey et al.(2015), Sugimoto et al. (2017), Feßler et al. (2018)
msr	plasmids	Feßler et al. (2018)
mef (A/E)/ C	MEGA element, transposons Tn*916* composite transposons, ICE*st3*, other ICEs and IncA/C plasmids	Chaffanel et al. (2015), Chancey et al.(2015), Sugimoto et al. (2017), Feßler et al. (2018)
*mph*G	plasmid	Sugimoto et al. (2017), Feßler et al. (2018)
ere	plasmid	Feßler et al. (2018)
Polymyxins (colistin)		
mcr family	IncP-1, IncFII, IncH, IncHI1, IncX4, IncI2 plasmids	Caniaux et al. (2017), Cannatelli et al. (2018), Li et al (2018)
*pmr*AB,C,F; *lpx*A,C,D; *mgr*B	chromosome, interruption by IS*Aba11*	Jaidane et al. (2015), Lean et al. (2015), Caniaux et al. (2017), Cannatelli et al. (2018), Lomonaco et al. (2018)
(Fluoro) quinolones (ciprofloxacin, nonfloxacin, enrofloxacin and levofloxacin)		
qnr family	SulA integron, IS2, IS26, ISEcI2Tn*3*, Inc A/C, H12, F, FII, L/M, N, R, Q, U, I1, X2, ColE1 plasmids and phages	Robicsek et al., 2006, Poirel et al., 2008, Strahilevitz et al., 2009, Karah et al., 2010, Rodríguez-Martínez et al., 2016
aac (6')-1b-cr	Inc FII, R and N plasmids	Poirel et al., 2008, Karah et al., 2010, Rodríguez-Martínez et al., 2016
*qep*A,	IS*29*, plasmids	Poirel et al., 2008, Rodríguez-Martínez et al., 2016
*oqx*AB	IS*26*, Tn*3*, IncX1, HI2, and F plasmids	Strahilevitz et al., 2009
*qac*BIII	FIA, FIB and FII plasmids	Strahilevitz et al., 2009
*gyr*A, *par*C, *rfa*D/E	chromosomal	Rodríguez-Martínez et al., 2016
Aminoglycosides (plazomicin)		
16 S rRNA transferase, *aac(2')-Ia*, *aph(2")-IVa*	chromosomal plasmids	Cox et al., (2018)
Ansamycins (kanglemycin A)		
No resistance		Mosaei et al. (2018)
Glycylcyclines (tigecycline)		
*acr*A/B	chromosome	Huang et al. (2017)
*ram*A, *rar*A	chromosome	Cha et al. (2018)
*omp*F	chromosome	Huang et al. (2017)
*opr*D	chromosome	Liu et al. (2016)
*rps*J	chromosome	Liu et al. (2016)
Aminomethylcycline (omadacycline)		
no resistance		Macone et al. (2014)
Lipopeptides (daptomycin)		
*mpf*R	chromosome	Bayer et al. (2013), Tran et al. (2015), Sabat et al. (2018)
Oxazolidinones (linezolid)		
cfr family, *optr*A, *poxt*A	Tn*6218*-like and different (unidentified) plasmids	Endimiani et al. (2011), Gu et al. (2014), Kuroda et al. (2018), Tyson et al (2018)

4 Mobility of genes conferring resistances to LRAs

4.1 Association of genes conferring resistance to LRAs with MGEs

Eventual association of genes conferring resistance to LRAs with MGEs will be illustrated in this section. Lateral transfer plays an important role in transmission of ARGs to other bacterial species, including the ones that are of clinical relevance. Association of genes conferring resistances to the group of LRAs (as presented in Table 1) with MGEs will be described in the next subsections and are summarized in Table 2.

4.2 Cephalosporins and carbapenems

Third, fourth and fifth generations of cephalosporins and carbapenems are classified as LRAs. However, considering the sharp increase in reports on carbapenem resistances over the last years, the focus in this document will be mainly on carbapenem resistances. Carbapenems belong to the group of β-lactam antibiotics that serve as LRAs in treatments of infectious diseases caused by bacteria that are resistant to first, second and third-line antibiotics (Chaudhary, 2016). Enzymes deactivating β-lactams by hydrolysis are named β-lactamases and carbapenemases are in fact β-lactamases capable to inactivate carbapenems. In total at least 1150 β-lactamases are nowadays known to be located on chromosomes, plasmids and/or transposons (van Hoek et al., 2011). Subdivisions can be made based on molecular identity of β-lactamases (so called Ambler classes; Bush and Jacoby, 2010; van Hoek et al., 2011. Kish, 2018) or on activity spectra (narrow, moderate, broad and extended spectrum β-lactamases [van Hoek et al., 2011]). The group of ESBLs confers resistances to penicillin, first, second and third generation cephalosporins and aztreonam, but not to carbapenems and are blocked in their activities by β-lactamase inhibitors (van Hoek et al., 2011). Using the Ambler classification, β-lactamases are divided over 4 groups (A through D) of which β-lactamases belonging to Ambler classes A, C and D utilize serine for β-lactam hydrolysis, whereas the ones belonging to class B use zinc, the so called class B metalloenzymes (Bush and Jacobi, 2010). Serine carbapenemases are present in Ambler classes A and D and metallo-carbapenemases in class B2 (Table 2). The most prevalent classes of carbapenemase genes are bla_{KPC} (*K. pneumoniae* metallo-β-lactamases), bla_{IMP} (imipenemase metallo-β-lactamases), bla_{NDM} (New Dehli metallo-β-lactamases), bla_{VIM} (Verona integron-encoded metallo-β-lactamases) and $bla_{OXA\text{-}48\text{-}like}$ (oxacillin carbapenemases) and these are the so called big five

group of ESBLs/ carbapenemases. Genetic loci encoding carbapenemases (three in class A, four in class D and three in class B2) are commonly located on, or closely associated with MGEs, including different classes of integrons and transposons and different incompatibility (Inc) groups of plasmids (Table 2). Two loci encoding carbapenemases, bla_{FRI} and bla_{NMC-A}, are located on chromosomes of, respectively, *Enterobacter cloacae* and *E. ludwigii* strains (Potter et al., 2016). Not all carbapenemase resistance phenotypes are conferred by β-lactamase activities, for example mutations in *arc*AB-*tol*C efflux pump genes and in *omp*C, F and K porin protein genes exist in enterobacterial species, whereas in *P. aeruginosa* other mutations causing resistance to carbapenems can be found (Potter et al., 2016). These mutations are, however, all chromosomally located and therefore not relevant in the context of this report. Overall, nearly all genes encoding carbapenemase activities are located on MGEs in Gram-negative (often enterobacterial) species (Table 2).

4.3 Glycopeptides

In 1997 the first report appeared on decreased susceptibility (increased minimal inhibitory concentration [MIC] of between 3 – 8 µg/ ml) to vancomycin in a MRSA isolate from Japan (Gardete and Tomasz, 2014). The resistance locus of these so-called vancomycin intermediate-resistant *S. aureus* (VISA) group of isolates was not found to be associated with MGEs. Later, however in 2002, the first reports appeared on vancomycin resistant *S. aureus* isolates showing MICs of 100 µg/ ml or higher and the resistance loci for this group of isolates were linked with transposon Tn*1546*, containing the vancomycin resistance locus acquired from *E. faecalis*, which was already described in early 1970s (Hegstad et al., 2010; Gardete and Tomasz, 2014). The resistance was based on cell wall precursor conversion from D-ala-D-ala to D-ala-D-lac, of which the last was less susceptible for the inhibitory activity of vancomycin in cell wall synthesis. The vancomycin *van*A resistance locus located on transposon Tn*1546* consisted of an operon with seven genes clustered in three loci, *van*XAH, *van*ZY and *van*SR (Table 2). The *van*XAH cluster of genes, encoding, respectively, a dipeptidase, a ligase and a dehydrogenase, was responsible for the cell wall precursor conversion from D-ala-D-ala to D-ala-D-lac. Of the other two clusters, *van*ZY contributed to high levels of vancomycin and low levels of teicoplanin resistance, whereas the *van*SR cluster was responsible for two component cascade signalling (Binda et al., 2014). In total 10 *van* operons (A, B, C, D, R, G, L, M and N) (Binda et al., 2014) and a recently discovered operon in *D. hafniense*, *van*I (Kruse et al., 2014), are nowadays described (Table 2). These operons

are responsible for different cell wall precursor modifications; expression from *van*A, B, D, M and I lead to D-ala-D-lac conversions, whereas expression from *van* C, E, G and L lead to D-ala-D-ser conversions. Remarkably, for all 10 operons resistance to vancomycin (MICs ranging from 2 - >256 µg/ ml) is higher than to teicoplanin (MICs ranging from 0.5 – 512 µg/ ml) (Binda et al., 2014). Vancomycin operons A, B, M and N are associated with ICEs, transposons and plasmids (Table 2). Therefore, most of the *van* operons must be considered as mobilizable resistance loci present among Gram-positive bacteria of clinical importance, but also among commensal Gram-positive species resident in the human gut microbiome. An exception is, however, the *van*I operon in *D. hafniense*, which does not contain the *van*H homologue inside the resistance gene cluster locus (Kruse et al., 2014). The physical location of the *van*H homologue outside the *van*I operon greatly hampers lateral transmission of the intact regulon to other (Gram-positive) species and thus lateral transmission of *van*I-mediated resistance must be considered as negligible.

4.4 Macrolides and ketolides

Erythromycin, originally isolated from a bacterium, *Saccharopolyspora erythreae*, became clinically available in 1952 for treatment of infections caused by Gram-positive bacteria such as *Enterococcus, Clostridium difficile*, but also by *Chlamydia trachomatis*. Later in the 1970s more acid stable derivatives of erythromycin, named clarithromycin, azithromycin and fidaxomycin, came on the market. Resistances have been found among clinically-relevant Gram-positive bacterial species such as *Staphylococcus* spp., *Streptococcus* spp., especially *S. pneunomiae* and *S. salivarius*, but also among Gram-negative (aquatic) bacteria such as *Vibrio, Photobacterium, Pseudoalteromonas* and *Shewanella* spp. (Table 2). Most likely, macrolide/ketolide resistant environmental bacteria, such as typical water-borne *Shewanella* species, were the progenitors of the resistant strains found among clinically relevant bacteria. Five macrolide resistance gene families have been found to date and all representatives are linked to MGEs. The *erm* family of macrolide resistance genes, all encoding 23S rRNA methylase, are linked to plasmids, transposable elements, genomic islands and a phage (ΦJN4341-pro). The *msr* family of resistance genes encode for ABC-F proteins conferring ribosomal protection to macrolides and these genes are associated with plasmids. The *mef* (encoding a macrolide cellular efflux pump) family of resistance genes are associated with the macrolide genetic assembly (MEGA), that on its turn carries conjugative Tn*916* (ICE*st3*) elements. Interesting from this perspective is that Tn*916* and derivatives are not able to

conjugate between *Streptococcus* species and because these species are known to be proficient for transformation, it seems likely that macrolide resistance acquisition to *S. pneumoniae* occurs via transformation (Chancey et al., 2015). IncA/C type of plasmids also carries *mef* genes and also a second macrolide resistance gene, *mph*G. This gene belongs to the class of *mpg* resistance genes and its gene product encodes for a macrolide phosphate transferase. Besides with IncA/C plasmids, this gene family is also associated with other plasmids and ICEs. The last macrolide gene family is *ere* group of macrolide resistance genes, which all encode for a macrolide esterase and this family of genes is commonly associated with plasmids.

4.5 Polymyxins

Colistin is used for antimicrobial treatments of infections caused by multidrug, and in particular by carbapenem-resistant Enterobacteriaceae, *A. baumannii* and *P. aeruginosa* strains and lineages (Caniaux et al., 2017). Colistin (polymyxin E) was reintroduced into medical healthcare as LRA to cure patients from infections caused by multi-resistant Gram-negative pathogens and for prophylaxis for oral and digestive tract decontaminations, in spite of its neurotoxic and nephrotoxic effects to humans (Hille et al., 2018). Until recently (2015), resistance to colistin was believed to be chromosomally borne until a plasmid-borne resistance to colistin, encoded from the *mcr*-1 locus, was described in *E. coli* and *K. pneumoniae* strains (Caniaux et al., 2017). The *mcr*-1 locus encodes a phosphoethanolamine transferase that adds ethanolamine residues to lipopolysaccharides (LPS) present in the outer membrane of Gram-negative bacteria thereby neutralizing the positive charge of LPS and thus decreasing the affinity of colistin to LPS (Caniaux et al., 2017). Mobilizable *mcr*-1 genes are presumed to originate from livestock where polymyxin was widely applied as supplement in cattle feed in the past (Hille et al., 2018). Nowadays eight *mcr* loci are reported in Gram-negative bacterial strains (Wang et al., 2018b) and all are associated with plasmids from different Inc groups (Table 2), among which a self-transmissible IncP-1 type of plasmid. Conjugation experiments with *Salmonella enterica* serovar Typhimurium and *K. pneumoniae* lineages containing *mcr*-1 genes revealed high *in vitro* transmission rates of between 8.2×10^{-5} – 2.07×10^{-1} per *E. coli* J53 recipient cell (Saavedra et al., 2017). Besides mobilizable *mcr* genes, other chromosomally-encoded loci are responsible for colistin resistance in *A. baumannii*, *P. aeruginosa* and Enterobacteriaceae (Table 2). These colistin-resistant phenotypes are caused by mutations or insertional inactivation by IS*Aba*1 in

lipopolysaccharide biosynthesis (*lpx*A, C and D), lipid A production (*pmr*CF) and two component transport system genes (*pmr*AB), and in *K. pneumoniae* chromosomal *mgr*B genes (Table 2). In spite of the fact that these genes are chromosomally encoded and presumed to be immobile, the presence of the IS*Aba1* element, possibly originating from a plasmid, indicate association with a MGE.

4.6 Quinolones

Nalidixic acid is a fully synthetic quinolone with bactericidal activity against *Enterobacteriaceae* and became clinically available in 1962 (Robicsek et al., 2006). Later generations of quinolones, with different additions of fluorine to the quinolone ring, were developed and these became available in the 1980s. The group of fluoroquinolones contains, amongst some other quinolones, ciprofloxacin, norfloxacin, enrofloxacin and levofloxacin. The molecular targets for quinolones in the bacterial cell are DNA gyrase and topoisomerase IV. There are different types of chromosomally-encoded resistances known and these resistances are based on mutations in the DNA-gyrase (*gyr*A) and the topoisomerase IV (*par*C) genes, and defects in lipopolysaccharide synthesis (*rfa*D and *rfa*E) genes (Table 2). Further, orthologues of the *qnr* gene (*qnr*A4, 5 and 6) are located on the chromosome of *Shewanella algae*, a bacterial species commonly found in aquatic environments. The *qnr* gene, of which its product confers protection for topoisomerases against quinolones, belongs to the group of the so called plasmid-mediated quinolone resistances (PMQRs). Most likely, quinolone resistance genes in *S. algae* strains must be considered as progenitors of PMQRs that are nowadays commonly found in many different bacterial species. The PMQRs are encoded by different gene families that include the *qnr* family of genes (*qnr*A, B, S, C, D and VC), *aac(6')-1b-cr* (an aminoglycoside modifying enzyme deactivating ciprofloxacin) and genes encoding efflux pumps (*qep*A, *oqx*AB and *qac*BIII) (Table 2). All three families are associated with MGEs including plasmids of different incompatibility groups (especially with a conjugative IncN type of plasmid [Karah et al., 2010]), integrons (class 1), transposable elements and phages (mainly *qnr*A and *qnr*S). Often quinolone resistance genes are linked to other resistance genes such as *amp*C and *bla* genes (conferring resistance against ampicillin, cephalosporins and carbapenems), and different groups of aminoglycoside resistance genes. The *aac(6')-1b-cr*-encoded acetyl-transferase confers co-resistance to tobramycin (Rodríguez-Martínez et al., (2016). The PMQRs are found among *Proteobacteria* and are most prominently present among Enterobacteriaceae.

4.7 Aminoglycosides

Streptomycin, originally isolated from *Streptomyces griseus*, belongs to the family of aminoglycosides. Other aminoglycosides are gentamicin, hygromycin, kanamycin and tobramycin and semi-synthetic compounds, amikacin, arbekacin, isepamicin and netilmicin. Aminoglycosides display bactericidal activities against aerobic Gram-negative species and also to some anaerobic and Gram-positive groups. Large groups of plasmid-mediated resistances are known (van Hoek et al., 2011) that can be classified into the three major groups of acetyltransferases, nucleotidyltransferases and phosphotransferases (all named aminoglycoside-modifying enzymes, AMEs). A different, chromosomally-encoded, 16S rRNA methyltransferases group of enzymes modifies the aminoglycoside target site, thus making aminoglycosides inactive for antimicrobial treatment. Resistances to aminoglycosides are well described in older literature and many of the aminoglycosides are clinically less relevant. However, next generations of aminoglycosides still are developed and the latest one is plazomycin (Cox et al., 2018; Kish, 2018). Most of the AMEs did not affect plazomycin in *E. coli* with the exception of *aac(2')-1a* and *aph(2'')-IVa* whose gene products decreased the potency of the antibiotic compound to a limited extent. However, the presence of the chromosomally-located 16S rRNA methyltransferase gene in *E. coli* completely abolished the antimicrobial effectiveness of plazomycin. Clonal variants of chromosomally encoded plazomycin resistant *E. coli* strains will be selected under aminoglycoside pressure in clinics and this will lead to further spread of plazomycin resistant bacteria.

4.8 Ansamycins

Rifamycin binds in the vicinity of the active site of the β-subunit of RNA polymerase and is blocking RNA elongation sterically. Semi-synthetic antibiotics were derived from rifamycin, of which rifampicin is the best known antibiotic compound. Resistances to rifamycin and rifampicin occur by spontaneous mutations in the RNA polymerase β-subunit making binding of both compounds to the RNA polymerase molecule impossible. Resistances to rifampicin are chromosomally encoded and spontaneous rifampicin-resistant derivative strains are commonly applied in scientific experiments. Rifampicin resistance is present in multi-resistant *Mycobacterium tuberculosis* strains, making rifampicin and other (semisynthetic) rifamycins non-functional for treatment of tuberculosis. However, a natural compound kanglemycin A, derived from a *Nocardia mediterranei* var. *kanglensis* strain, binds to the

same target site on the RNA polymerase as rifampicin, even in Gram-positive bacteria and multi-resistant *M. tuberculosis* strains that were resistant against rifampicin, making kanglemycin A a potent agent for suppression of growth of rifampicin resistant bacteria (Mosaei et al., 2018). Kanglemycin A and semi-synthetic derivatives of this compound make ansamycins effective again for antimicrobial treatment of infections caused by multi-resistant *M. tuberculosis* strains.

4.9 Tetracyclines/ Glycylcyclines/ Aminomethylcyclines

Widespread resistances exist against the older tetracyclines: tetracycline, doxycycline and minocycline. New 'tetracyclines' have been developed and recently approved and these include glycylcyclines, specifically tigecycline, fluorocyclines, including eravacycline, and aminomethylcyclines, including omadacycline. Resistances to tigecycline were reported in (carbapenem-resistant) *Enterobacter* spp., *E. coli* and *A. baumannii* strains (Table 2). Resistances were all located on chromosomes and no associations with MGEs were reported. Resistances were based on point mutations and homologous recombination in efflux pump (*acr*A/B), transcriptional regulator (*ram*A and *rar*A), outer membrane (*omp*F) protein and porin (*opr*D), ribsomal S10 protein (*rps*J), and benzoate degradation genes. Resistance to omadacycline officially has not been reported to date (Macone et al., 2014), but increased minimal inhibitory concentrations (MIC) for omadacycline has been reported in clinically relevant bacteria and often these resistances were correlated with resistances to tetracycline. Omadamycin is effective against MRSA and Gram-negative species such as *E. coli, H. influenzae* and *K. pneumoniae,* even in strains that carry the 'classical' tetracycline resistance mechanisms such as ribosomal protection and efflux pump determinants.

4.10 Lipopeptides

Daptomycin, approved as antibiotic by the US Food and Drug Administration in 2003, is used as frontline (last resort) agent against MRSA and against methicillin-sensitive *S. aureus* in patients that possess allergies against β-lactams (Bayer et al., 2013). The working mechanism of daptomycin is distinct from most other antibiotics as it causes rapid depolarization of the bacterial cell membrane and ultimately leading to cell death, however without causing cell lysis. Chromosomally-borne daptomycin resistances have been reported to date (Table 2).

Best described is the multiple peptide resistance factor (*mpf*R) gene involved in cell wall and membrane homeostasis. Comparisons between two clinical *S. aureus* strains resistant to daptomycin and one *S. aureus* strain susceptible to this antimicrobial agent on the basis of whole genome DNA sequence and RNAseq analyses revealed co-expression of core genome genes that correlate with daptomycin resistance (Sabat et al., 2018).

4.11 Oxazolidinones

From 2001 on, linezolid has become an alternative to break vancomycin resistance in therapies against (vancomycin-resistant) MRSA in patients with lung diseases and cystic fibrosis (Gu et al., 2012). However, one year after its approval, resistances against linezolid were already reported, especially in specific clonal types (sequence type [ST] 5) of *S. aureus* (Endimiani et al., 2011; Gu et al., 2012). Resistances to linezolid were caused by mutations in the 23S ribosomal RNA binding site, in the L3 and L4 ribosomal proteins responsible in peptide translocation and by a plasmid-borne ribosomal methyltransferase encoded from the *cfr* gene (Endimiani et al., 2011; Gu et al., 2012). A variant of the plasmid-borne *cfr* gene, *cfr*B, was shown to be located on a transposon Tn*6218*-like element in *E. faecalis* that was 99% identical to a homologue present in *C. difficile* (Kuroda et al., 2018). Further, one (*optr*A) out of two additional linezolid resistance genes, and both conferring protection to ribosomal genes, were found to be located on a hitherto unidentified plasmid (Kuroda et al., 2018). Both *cfr* and *optr*A genes were located on plasmids of (vancomycin-resistant) *E. faecium* and *E. faecalis* strains (Lazaris et al., 2017; Tyson et al., 2018). One plasmid of 73 kb in size and some other plasmids present in *E. faecium* strains, all containing *cfr* and *optr*A genes, also possessed loci conferring resistance to other antibiotics. One novel plasmid of 8 kb in size from an MRSA strain contained the *cfr* gene (Lazaris et al., 2017; Tyson et al., 2018). This information demonstrates the potential for lateral transmission of linezolid resistance genes among Gram-positive species (Table 2).

4.12 Relevance of ARG association with MGEs in the context of this report

Genes conferring resistance to LRAs commonly are associated with different types of MGEs such as integrons, IS elements and transposons, ICEs, phages and different types of plasmids as shown in Table 2. There is no preference of these type of ARGs for any MGE in particular.

Further, the ICEs, phages and plasmids will have different transfer rates and host ranges, which are, in most cases, even unknown and local reigning circumstances (nutrient availability, attachment and bacterial incorporation in biofilms) will further influence the possibilities for LGT. It is therefore not possible to provide any quantitative prediction on ARG transmission via these elements in bacterial strains that are used under contained circumstances, for example in the laboratory.

5 Conclusions

- Resistances to antibiotics are common in all natural environments including permafrost samples dating from before the antibiotic era and from the ones that thus far remained undisturbed by human influences.
- Recruitment, clustering, mobilization and lateral transmission of ARGs will occur in all environments (clinical and natural) under antibiotic selective pressure.
- Antibiotic pressure selects for antibiotic resistant lineages among bacteria present in clinical and non-clinical (e.g. sewage water and manure) environments.
- Environmental bacterial strains carrying ARGs associated with MGEs are in principle always capable to transmit antibiotic resistances to clinically relevant bacterial species.
- ARG recruitment and clustering are long-term processes that take place in the environment, but ARG transmission, via conjugation, transformation and transduction, are processes that can take place in the short time frame realistic for laboratory work.
- Chances on lateral transmission of antibiotic resistances in bacterial strains, that are not associated with any type of MGE in their genomes, must be considered as negligible.

6 Recommendations and decision model

For risk assessment analysis (Fig. 2), it is important to know which type of ARGs is present in bacterial strains. Preferably, presence of ARGs in bacterial strains must be determined via genotypic (full genome sequence analysis) and sometimes phenotypic screening. The most up to date list of 'critically important antibiotics' of the WHO (the most up to date list for now is the 2016 version) can be taken as guideline for the clinical relevance of ARGs. If resistances against critically important antibiotics are present in bacterial strains, then information needs to be provided about the localization on the genome (chromosome or on extrachromosomal elements such as plasmids and phages) and eventual association with (other) MGEs. In case ARGs are associated with MGEs, then there always will be a considerable chance on intra- and intergenomic recombination and on lateral transmission of ARGs either via conjugation (plasmids or ICEs), transduction (phages) and/ or transformation (under the premises that bacteria are naturally competent and, for chromosomal DNA transfer, that there is sufficient homology between donor DNA and DNA sequences present in the genome of the receiving strain). The decision model shown in Fig. 2 can also be applied for multiple genes conferring resistance to one particular class of antibiotics, as is the case for glycopeptide (vancomycin - teicoplanin) resistance. All genes responsible for vancomycin - teicoplanin resistance must be clustered in a distinct operon on a MGE as is the case with the *van*A and *van*B operons. This was not the case for the *van*H homologue, that was located outside the *van*I operon in *D. hafniense* (Kruse et al., 2014). In this particular case, lateral transmission of the complete set of genes responsible for conferring resistance to glycopeptides must be considered as low.

MGE types and their relevance in lateral transmission of associated ARGs to other bacteria are categorized in Table 3. The classification shown in Table 3 is based on self-control in transmission of the MGE, (estimated) transfer frequencies and host ranges. The first category consists of (conjugative) self-transmissible plasmids, either with narrow (Inc F, H, I) or broad host ranges (Inc N, P, W). Transmission by this type of MGE is self-controlled by making use of only two parental (donor and recipient) cell types. Transfer frequencies of self-transmissible plasmids can be very high, to up to 10 – 50% of the recipient cell population (Heuer and Smalla, 2012). The second category consists of plasmids that cannot regulate their own transmission and that need help from another plasmid present in a distinct donor cell population (donor helper cells) for transmission of ARGs, as is the case for the group of conjugative but non-self-transmissible (mobilisable) plasmids such as IncQ plasmids. These

type of plasmids occasionally can have high transfer frequencies and can reach broad species spectra, but conjugation of these type of plasmids rely on the presence of a self-transmissible plasmid present in a second donor cell population. Expectedly, the frequency in occurrence of triparental matings will be lower than of biparental matings with self-transmissible plasmids in nature. The reason that plasmids with broad and narrow host ranges are not classified into separate categories is because predicted host ranges of particular Inc group of plasmids substantially differ from experimentally determined host ranges (Heuer and Smalla, 2012; Suzuki et al., 2010), indicating the great uncertainty that still exist on actual host ranges of different Inc groups of plasmids. The third category consists of phages and often there is a high specificity of phages for their hosts. Transduction frequencies can be high, to up to 10^{-2} per recipient cell. The fourth category consists of 'naked' DNA, and for transduction, competence for DNA uptake in bacteria is required and also sufficient homology for DNA integration in case of extracellular chromosomal DNA fragments, which is not the case for plasmid and phage DNA. The fifth category consists of transposons/ IS elements and for LGT, transposition events to other MGEs must take place in first instance, before actual transmission to other bacteria can occur.

A ranking on the basis of possibility for transmission of ARGs to other bacteria is made in Table 3. Congruent data on frequency of transmission and host range of the MGEs where ARGs are located on is only provided in exceptional cases in the literature. Transfer frequencies of ARGs located on MGEs can be very high, especially when ARGs are located on plasmids. Also, the host range can be broad and IncP-1 type of plasmids commonly confer high transfer rates to broad bacterial species spectra and this type of plasmid can be considered as a 'worst case' in the transmission of ARGs in general. For example, *mcr* genes conferring resistance to colistin was located on an IncP-1 plasmid (Li et al., 2018; Table 2). Other MGEs confer lower transmission frequencies to ARGs and also bacterial host ranges are narrower. LGT via conjugation (transfer via single or double donor strains), transduction and transformation (categories 1 - 4) can occur in relatively short time frames that are realistic under confined, laboratory, circumstances (within one to several hours). Transposition to MGEs followed by LGT via other MGEs are distinct processes taking place in sometimes different bacterial genomes and therefore the expected time for ARGs to be transmitted via transposons/ IS elements to other bacteria will be longer than for the MGEs described in categories 1 – 4. Also, the expected LGT frequencies of transposons/ IS elements (transposition frequency to the MGE times transfer frequency of the MGE with transposon to other bacteria) will be much lower than for the other LGT types shown in Table 3. Therefore

LGT category 5 is lowest in ranking, indicating that eventual occurrence of LGT via bacterial strains containing ARGs located on transposons or adjacent to IS elements must be expected to be very low, although, LGT via these MGEs cannot totally be ruled out.

In summary, contamination of workers with bacterial strains carrying ARGs do not directly affect the health status of these individuals. Therefore, antibiotic resistance in bacteria must be considered as a ‘hazard’ and not as ‘risk’. Only under specific circumstances where ARGs are transmitted to bacteria causing infections in humans, impact on health might be expected, in case antibiotic treatment of infections fail. Dissemination of ARGs via unintended releases from the contained area (laboratory) to the environment may lead to further spread of these ARGs to other bacteria via LGT. However, that these incidental releases of bacterial strains carrying ARGs would lead to increased risks for antibiotic resistances in clinically relevant bacteria seems unlikely. For dissemination of ARGs to other bacteria, antibiotic selection pressure is needed and as long as selective pressure is absent, no specific stimulation for growth of antibiotic resistant bacteria can be expected.

Table 3. Typing of LGT systems, based on bacterial LGT mechanisms, into separate categories dependent on self-control in MGE transmission, transfer frequency and host range.

LGT type	MGE type	Criteria
1	**Self-transmissible plasmids, some ICEs**	**Biparental conjugation** Transfer frequency to up to 10 – 50% / recipient cell Transfer to broader species spectra One single donor strain needed No other MGEs needed for transmission Time frame for transmission, one – several hours
2	**Mobilisable plasmids, some ICEs**	**Triparental conjugation** Transfer frequency to up to 10 – 50% / recipient cell Transfer to broader species spectra Other plasmid in a separate donor population needed Time frame for transmission, one – several hours
3	**Phages**	**Transduction** Transfer frequency to up 10^{-2}/ recipient cell High specificity for host strains, Time frame for transmission, one – several hours
4	**'Naked' DNA (in the form of chromosomal, plasmid or phage DNA)**	**Transformation** Competence of the recipient strain needed Transfer efficiency depends on DNA type and can be up to 10^{11} transformants/ µg plasmid DNA Time frame for transmission, one – several hours
5	**Transposons and IS elements**	**Transposition** Transmission to other MGEs (plasmid, ICE, phage) required before transmission to other species is possible. Likely does not fit in time frame for laboratory work

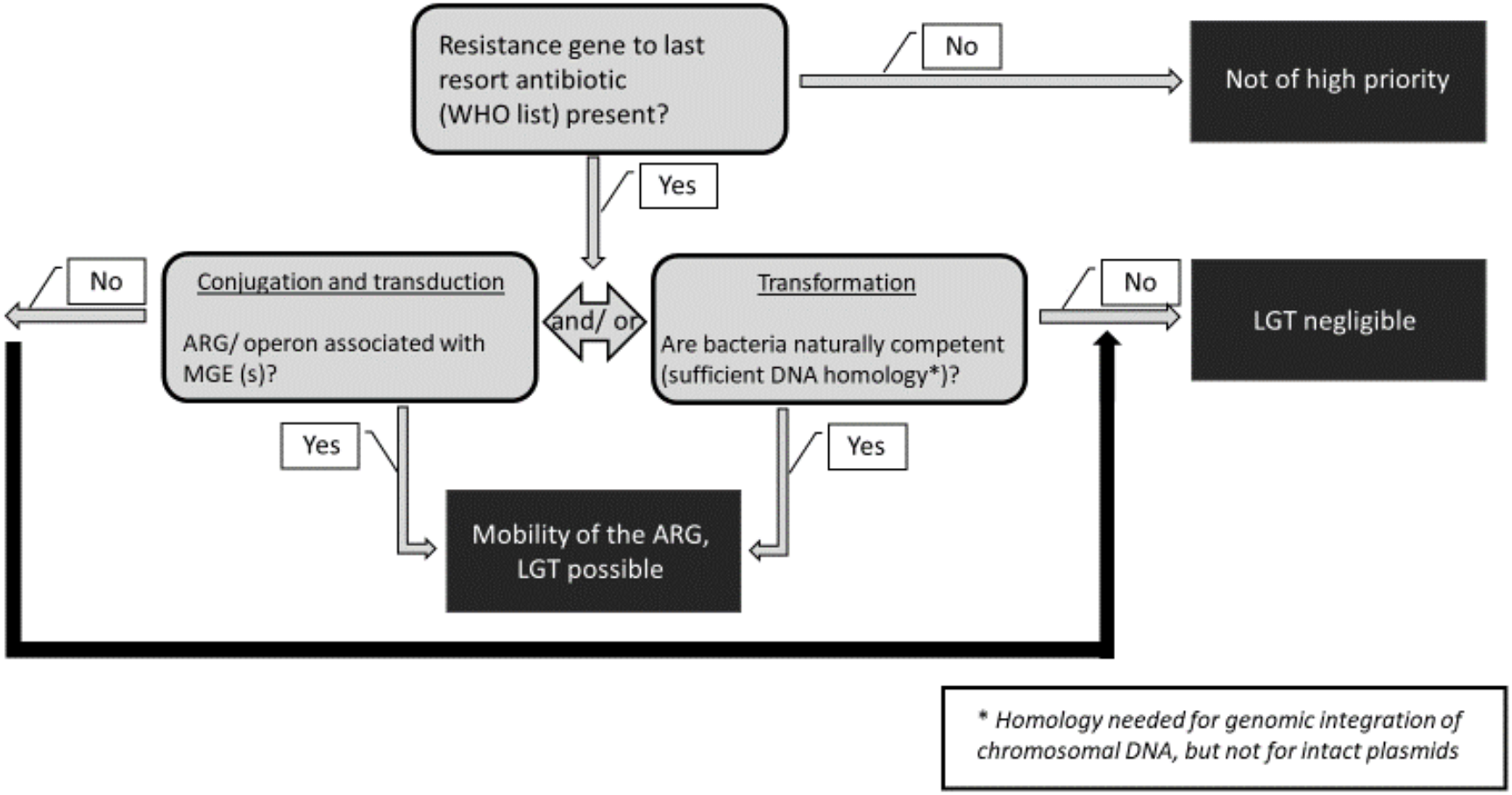

Fig. 2. Decision model for classification of bacterial strains containing resistance genes towards LRAs based on associations with MGEs.

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Supplementary material

Strategy for bibliographic searches in library databases

For this report, a semi-systematic literature search was performed according to a three-step approach (FigS1). Therefore, relevant publications and reports were automatically searched in databases and then further selected out by hand on the basis of relevance within the framework of the report. In the first step, a literature search was conducted only using the term 'antibiotic resistance' in titles, abstracts and keywords of all peer-reviewed publications, reports and symposium abstracts present in five library databases. Last search was done on 20 December 2018. These searches resulted in numbers of between 37,279 (CAB abstracts) – 218,640 (PubMed) references. Restricting the database searches to publications from the year of 2010 onwards only, still by making use of the same definition as query, resulted in numbers of references of between 21,961 (CAB abstracts) – 88,750 (PubMed). These numbers illustrate the vast number of scientific reports on the topic of 'antibiotic resistance' that are present in general (Scopus, Web of Sciences), biomedical (MedLine, PubMed) and applied life sciences (CAB abstracts) library databases.

As second step, definitions for queries among the same databases were therefore restricted by making use of combinations in search terms, by combining the term 'antibiotic resistance' with 'mobilome', 'resistome', 'microbiome', 'mobile genetic element', 'pangenome', 'gene', 'self-transmissible' and/ or 'host range'. Further specifications on search definitions, based on tolerated variations in definitions and maximum number of words between definitions are shown in Table S1. For all combinations, queries were applied to these five databases, later followed by setting a restriction for reports that appeared after 2010 only. Based on all attempted combinations, the selected numbers of non-duplicate publications were between 22 (antibiotic resistance AND pangenome AND mobile genetic element as query) and 656 (antibiotic resistance AND resistome as query) (Table S1). Some of the queries still delivered too high numbers for selecting out duplicate references by hand (antibiotic resistance AND gene), (antibiotic resistance AND microbiome), whereas for other queries the variation in search terms resulted in about the same number of references, which was the case for the combinations of 'antibiotic resistance AND pangenome with and without the term mobile genetic element' and for 'antibiotic resistance AND gene AND self-

transmissible AND host range, with or without the term ‘mobile genetic element’. Using the combinations of search terms with ‘mobilome’, ‘resistome’ and ‘pangenome’, (almost) only references from 2010 and later were obtained, demonstrating that most genomic and metagenomic-based reports related to antibiotic resistance appeared after that time (Fig. S2)

As third step, selected references (last column in Table S1) were screened for reports on ‘last resort antibiotics’. This group encompassed the classes of: third, fourth and fifth generations of cephalosporins and carbapenems, glycopeptides, macrolides and ketolides, polymyxins, quinolones, aminoglycosides, ansamycins, glycylcyclines and aminomethylcyclines, lipopeptides and oxazolidinones. Peer-reviewed publications, based on associations between LRAs and MGEs and references within these publications, were further selected out by hand and a list of genes conferring resistances towards LRAs, eventually associated with MGEs, was prepared. This list was shortened on the basis of relevance to the aim of the study, using the advices from two leading scientists in the fields of clinical and veterinary bacteriology (see acknowledgements). Most relevant resistance genes of LRAs with eventual associations with MGEs are shown in Table S2.

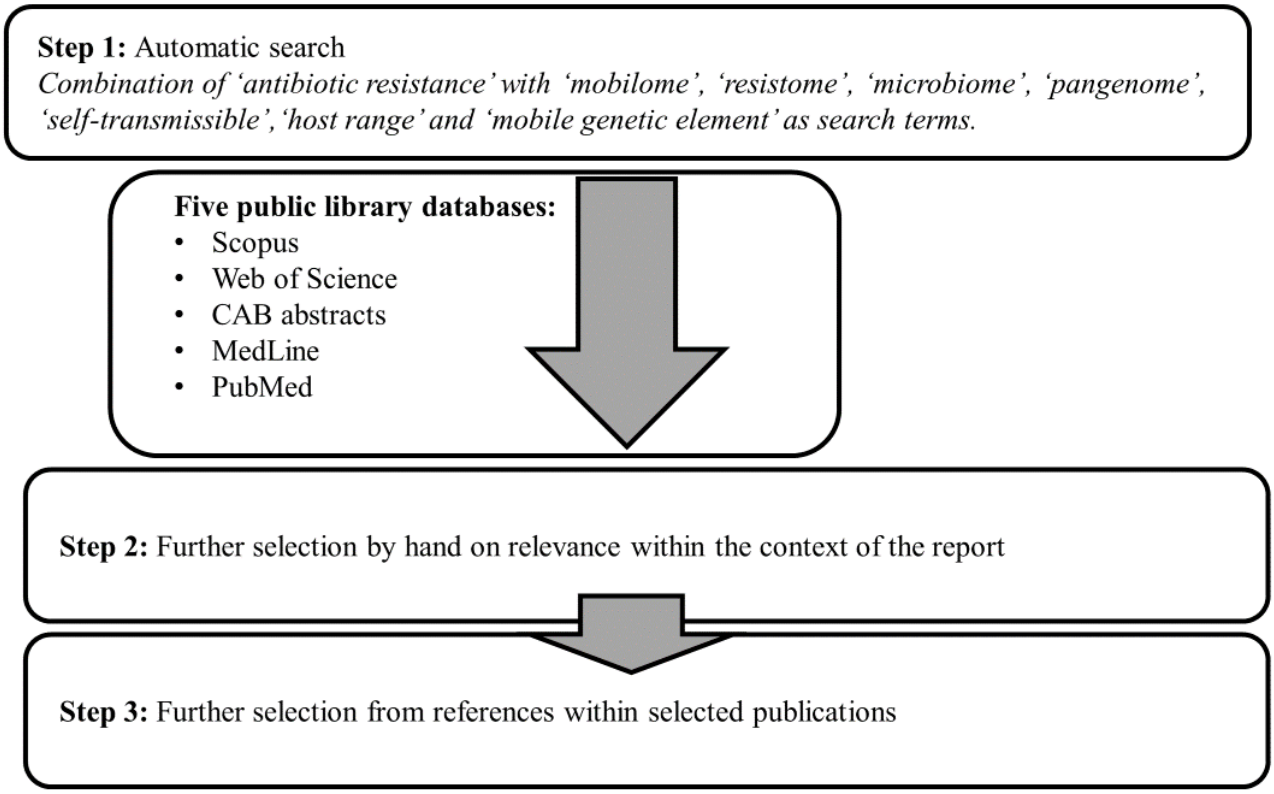

Fig. S1. Schematic overview of the semi-systematic literature search approach conducted in this report.

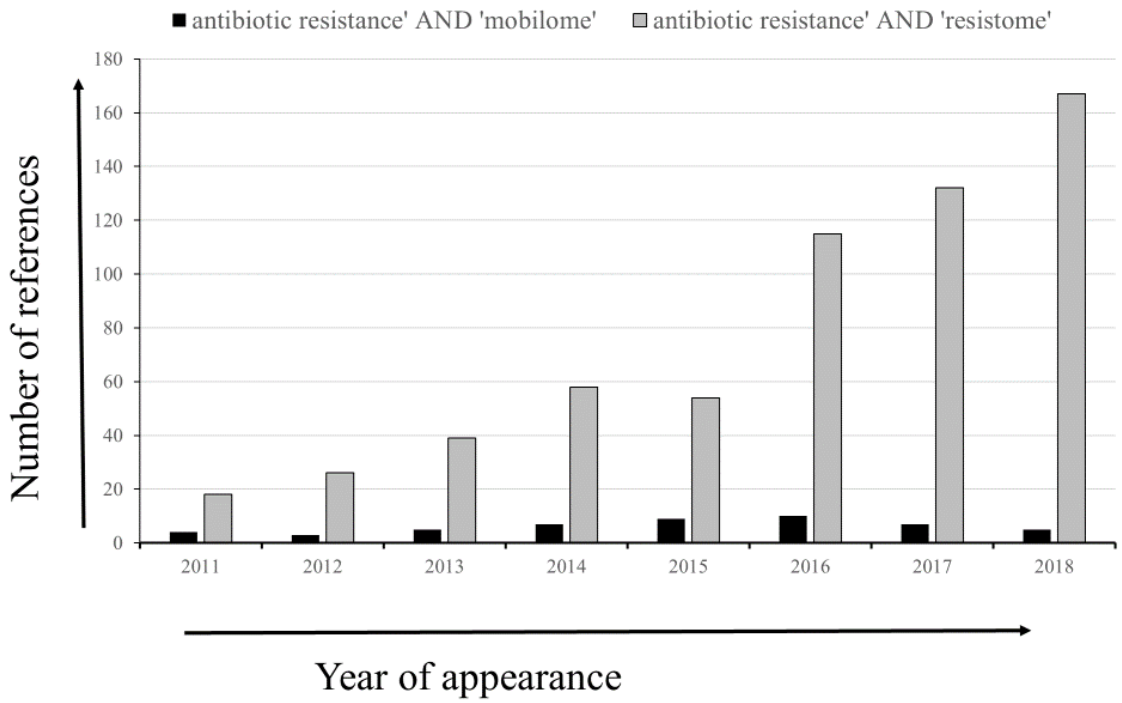

Fig. S2. Number of publications that appeared after the year of 2010, obtained from five library databases using a semi-systematic search approach based on combinations of the term ‘antibiotic resistance’ with ‘mobilome’ and ‘resistome’ in queried search definitions.

Table S1. Maximum tolerated variations in definitions and number of words is search definition variants based on 'antibiotic resistance'.

Concept	Line	Query
Antibiotics resistance	1	TITLE-ABS-KEY(Antibiotic* W/3 resistan* OR anti-biotic* W/3 resistan* OR antimicrobial W/3 resistan* OR anti-microbial W/3 resistan* OR multidrug W/3 resistan* OR MDR OR multi-drug W/3 resistan* OR "multiple drug" W/3 resistan* OR multiple-drug W/3 resistan* OR "totally drug resistan*" OR "extensively drug resistan*" OR XDR OR "pandrug resistan*" OR "pan drug resistan*" OR PDR OR "plasmid mediated" W/3 resistan*)
mobilome	2	TITLE-ABS-KEY(mobilome)
resistome	3	TITLE-ABS-KEY(resistome)
microbiome	4	TITLE-ABS-KEY(microbiome)
pangenome	5	TITLE-ABS-KEY(pangenome)
gene	6	TITLE-ABS-KEY(gene)
self-transmissible	7	TITLE-ABS-KEY(self-transmissible OR conjugative)
host range	8	TITLE-ABS-KEY(Host W/3 range OR hostrange)
mobile genetic element	9	TITLE-ABS-KEY("mobile genetic element" OR "insertion element" OR transposon OR retrotransposon OR "transposable element" OR retroelement OR plasmid OR phage OR prophage OR bacteriophage OR resistome OR "r factor" OR "resistance factor")
Antibiotics resistance AND mobilome	10	1 AND 2
Antibiotics resistance AND resistome	11	1 AND 3
Antibiotics resistance AND microbiome	12	1 AND 4
Antibiotics resistance AND microbiome AND Mobile genetic element	13	1 AND 4 AND 9
Antibiotics resistance AND pangenome	14	1 AND 5
Antibiotics resistance AND pangenome AND Mobile genetic element	15	1 AND 5 AND 9
Antibiotics resistance AND gene	16	1 AND 6
Antibiotics resistance AND Gene AND Self-transmissible	17	1 AND 6 AND 7
Antibiotics resistance AND Gene AND Self-transmissible AND Host range	18	1 AND 6 AND 7 AND 8
Antibiotics resistance AND Gene AND Self-transmissible AND Host range AND mobile genetic element	19	1 AND 6 AND 7 AND 8 AND 9

Table S2. Number of publications obtained from five library databases using a semi-systematic search approach based on variations on the term 'antibiotic resistance' in queried search definitions.

Queried search definition	Number of references (all publications until 31-12-2018/ publications from 1-1-2010 - 31-12-2018 only)					
	Scopus	Web of Science	CAB Abstracts	MedLine	PubMed	Total (all publications)
Antibiotic resistance	186,367/ 80,579	149,951/ 87,963	37,279/ 21,961	100,160/ 50,018	218,640/ 88,750	*
Antibiotic resistance AND Mobilome	36/ 35	41/ 40	10/ 10	22/ 22	34/ 34	51
Antibiotic resistance AND Resistome	442/ 426	526/ 502	131/ 125	262/ 252	434/ 414	656
Antibiotic resistance AND Microbiome	1,004/ 997	732/ 726	153/ 151	458/ 453	549/ 544	*
Antibiotic resistance AND Microbiome AND Mobile genetic element	219/ 219	165/ 165	41/ 41	130/ 130	135/ 135	296
Antibiotic resistance AND Pangenome	29/ 29	34/ 34	3/ 3	14/ 14	26/ 26	*
Antibiotic resistance AND Pangenome AND Mobile genetic element	14/ 14	14/ 14	1/ 1	6/ 6	12/ 12	22
Antibiotic resistance AND Gene	50,772/ 28,605	47,688/ 27,895	11,006/ 7,721	29,978/ 16,544	36.554/ 17,279	*
Antibiotic resistance AND Gene AND Self-transmissible	1,686/ 853	1,464/ 881	323/ 194	901/ 502	1.101/ 570	*
Antibiotic resistance AND Gene AND Self-transmissible AND Host range	118/ 56	136/ 74	22/ 11	63/ 33	71/ 37	*
Antibiotic resistance AND Gene AND Self-transmissible AND Host range AND Mobile genetic element	116/ 54	124/ 67	20/ 10	61/ 32	67/ 35	183