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REVIEW ARTICLE

Colistin and tigecycline resistance in carbapenemase-producing Gram-negative bacteria: emerging resistance mechanisms and detection methods

J. Osei Sekyere[1], U. Govinden[1], L.A. Bester[2] and S.Y. Essack[1]

1 Antimicrobial Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
2 Biomedical Resource Unit, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa

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Correspondence
Usha Govinden, Antimicrobial Resistance Unit, Discipline of Pharmaceutical Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa.
E-mail: govindenu@ukzn.ac.za

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Summary

A literature review was undertaken to ascertain the molecular basis for tigecycline and colistin resistance mechanisms and the experimental basis for the detection and delineation of this resistance particularly in carbapenemase-producing Gram-negative bacteria. Pubmed, Google Scholar and Science Direct were searched with the keywords colistin, tigecycline, resistance mechanisms and detection methods. Trans-complementation and comparative MIC studies, mass spectrometry, chromatography, spectrofluorometry, PCR, qRT-PCR and whole genome sequencing (WGS) were commonly used to determine tigecycline and colistin resistance mechanisms, specifically modifications in the structural and regulatory efflux *(acrAB, OqxAB, kpgABC adeABC-FGH-IJK, mexAB-XY-oprJM* and *soxS, rarA robA, ramRAB marRABC, adeLRS, mexRZ* and *nfxb*) and lipid A (*pmrHFIJFKLM, lpxA, lpxC lpxD* and *mgrB, pmrAB, phoPQ,*) genes respectively. Mutations in the ribosomal 16S rRNA operon *rrnBC,* also yielded resistance to tigecycline through target site modifications. The *mcr-1* gene conferring resistance to colistin was identified via WGS, trans-complementation and a murine thigh infection model studies. Common detection methods are mainly antibiotic sensitivity testing with broth microdilution while molecular identification tools are mostly PCR and WGS. Spectrofluorometry, MALDI-TOF MS, micro-array and real-time multiplex PCR hold much promise for the future as new detection tools.

Introduction

The proliferation of extended spectrum β-lactamases (ESBLs)—notably TEM, SHV and CTX-M among Gram-negative bacteria, mediated through promiscuous conjugative plasmids, transposons and integrons ushered the carbapenems into clinical practice (Sekyere *et al.* 2016). Consequently, carbapenems became the main reserve antibiotics used for difficult-to-treat ESBL-mediated infections, invariably increasing the volume of carbapenems used in clinical medicine worldwide (Van Boeckel *et al.* 2014; Sekyere *et al.* 2016; Osei Sekyere *et al.* 2015). This has resulted in evolutionary adaptations in Gram-negative bacteria under carbapenems selection pressure (Livermore and Woodford 2006). Through porin mutations, efflux upregulation and carbapenemase production, Gram-negative bacteria have evaded the lethal effects of carbapenems, rendering them ineffective (Livermore and Woodford 2006; Sekyere *et al.* 2016). Within this decade, notorious carbapenemases like the new-Delhi metallo-β-lactamases (NDMs), OXA-48-like carbapenemases and *Klebsiella pneumoniae* carbapenemases (KPCs) have spread quickly throughout the world, and have become endemic in the Indian subcontinent and in the Mediterranean, Southern and Northern American regions respectively (Munoz-Price *et al.* 2013; Dortet *et al.* 2014; Nordmann and Poirel 2014). Especially borne on IncF-type plasmids (Carattoli 2009) and transposons, carbapenemases have become established in *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Kl. pneumoniae*,

Escherichia coli and *Enterobacter* spp., (Munoz-Price *et al.* 2013; Nordmann and Poirel 2014; Sekyere *et al.* 2016) and have been implicated in high mortalities (Capone *et al.* 2013; Falagas *et al.* 2014), posing a major threat to the management of clinical infections.

To contain the spread of carbapenem-resistant Gram-negative bacteria, anal screening and isolation of patients, rapid detection methods and other infection control methods were adopted (Center for Disease Control and Prevention (CDC) (2010, 2013); Osei Sekyere *et al.* 2015). Carbapenemase detection methods include multiple chromogenic and nonchromogenic screening media, the modified Hodge's test, culture-based double disc synergy and disc-inhibitor combination assays, spectrophotometry, biochemical tests (like the RAPIDEC® CARBA NP and the Rapid CARB screen kits) matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), micro-arrays, PCR-based assays and next generation sequencing (Osei Sekyere *et al.* 2015). Notwithstanding these interventions, reports of carbapenemase-mediated resistance continue to increase globally, (Nordmann and Poirel 2014; Sekyere *et al.* 2016) leading clinicians to resort to other antibiotics with therapeutic success against carbapenemase-producing Gram-negative bacteria.

Subsequently, colistin (polymyxin E) and tigecycline have become the antibiotics of last resort for carbapenemase-producing Gram-negative bacteria, effectively replacing carbapenems as a reserve antibiotic (Monaco *et al.* 2014; Olaitan *et al.* 2014; Van Boeckel *et al.* 2014). Increasing colistin and tigecycline consumption has been concurrent with increasing reports of tigecycline and colistin resistance, especially during therapy (Rodriguez-Avial *et al.* 2012; Brink *et al.* 2013). Already, clinical outbreaks involving colistin resistant KPC-producing *Kl. pneumoniae* have been reported in the United States and Italy with worrying recurrence (Marchaim *et al.* 2011; Mammina *et al.* 2012; Capone *et al.* 2013; Monaco *et al.* 2014). Increasing nonsusceptibility to colistin and tigecycline during colistin and tigecycline monotherapy (Rodriguez-Avial *et al.* 2012; Hong *et al.* 2013) has engendered the use of colistin and/or tigecycline in double and triple combinations with a carbapenem, an aminoglycoside (amikacin, gentamycin and tobramycin), rifampicin, fosfomycin or fluoroquinolone (Mezzatesta *et al.* 2011; Tascini *et al.* 2013; Stein *et al.* 2015). These combination therapy approaches have, however, been unable to achieve 100% clinical cure rates in infected patients (Neuner *et al.* 2011; Ku *et al.* 2012; Capone *et al.* 2013). Tigecycline and colistin combination therapy has been shown to be antagonistic in *Ac. baumannii*, specifically at low concentrations in some studies (Albur *et al.*, 2012; Cikman *et al.*, 2015) but synergistic in others (particularly in Enterobacteriaceae) (Ku *et al.* 2012; Betts *et al.*, 2014). Tigecycline with doripenem (Hong *et al.* 2013; Dinc *et al.*, 2015) or amikacin (Dinc *et al.*, 2015) has been very promising.

It is thus imperative to develop methods to rapidly detect tigecycline and colistin resistance in Gram-negative bacteria based on a comprehensive understanding of the underlying resistance mechanisms. There are several reviews addressing tigecycline and colistin resistance in terms of their epidemiology and/or resistance mechanisms (Sun *et al.* 2013; Ah *et al.* 2014; Olaitan *et al.* 2014). However, none of these reviews provide the experimental studies used to detect the various colistin and tigecycline resistance mechanisms. We therefore present a review of established and emerging resistance mechanisms of tigecycline and colistin, their underlying experimental support and the existing methods used to detect their resistance in Gram-negative bacteria. We further propose tools that could potentially be developed to enhance the rapid, reliable detection of colistin and tigecycline resistance in the clinical laboratory.

Experimental basis of colistin and tigecycline resistance mechanisms

Methods that have thus far been used to understand tigecycline and colistin resistance mechanisms include phenotypic approaches like culture-based tests, spectrophotometry, immunological tests (ELISA), chromatography, biofilm formation assays and genomic/molecular methods like PCR and whole genome sequencing (WGS). Experimental procedures and the resistance mechanisms are discussed separately for colistin and tigecycline hereunder:

Colistin (polymyxins)

Polymyxins are polypeptide antibiotics discovered in 1947 from *Bacillus polymyxa* (Falagas *et al.* 2005). They comprise of five different structural analogues classified into groups A to E, albeit polymyxins B and E (colistin) are the most clinically important (Falagas *et al.* 2005). Reports of nephrotoxicity from various parts of the world led to their neglect, specifically colistin, in the 1980s. Colistin is available commercially as the sulphate and colistimethate sodium salts for treating infections of both Gram-negative and Gram-positive bacteria; the colistimethate sodium salts are less toxic and potent than the sulphate salts, which are administered orally or topically (Falagas *et al.* 2005). Polymyxins share the same mechanisms of action and studies analysing colistin resistance mechanisms are commonly undertaken with polymyxin B (Moffatt *et al.* 2010; Beceiro *et al.* 2014).

Resistance to colistin is mediated mainly through lipid A structural adjustments, resulting from the addition of phosphoethanolamine (Pet) and 4-amino-4-deoxy-L-arabinose (L-Ara-4N) to the lipid A moiety on the surface membrane; these additions make lipid A less cationic such that the anionic colistin is unable to bind and initiate membrane lysis (Ah *et al.* 2014; Olaitan *et al.* 2014). Genes that encode enzymes involved in the synthesis of lipid A are the *pmrHFIJFKLM* (also known as *arnBCADTEFpmrE*) in Gram-negative bacteria in addition to *lpxA, lpxC* and *lpxD* in *Ac. baumannii.* These genes are in turn regulated by *pmrAB, phoPQ* and *mgrB* genes. Hence, mutations in any of these genes result in a defect in lipid A synthesis and/or addition of L-Ara-4N or PEt to lipid A, leading to colistin resistance through reduced anionic charges. Recently, a plasmid-borne Pet transferase, *mcr-1*, has been identified in China, Malaysia and Laos, in swine, raw meat and hospitalized patients (Liu *et al.* 2015).

Capsules mediate resistance to colistin

The role of capsules in colistin resistance was determined with capsular and noncapsular clinical *Kl. pneumoniae* 52145 (serotype 01:K2) isogenic mutants 52K10 and 52021 respectively. The optical densities of suspensions of each mutant that had been incubated with polymyxin B were measured. Concurrently, 1-naphthylamide (NPN) was added to 52K10 and 52145 bacterial suspensions with 5 μ mol l^{-1} carbonyl cyanide m-chlorophenylhydrazone (CCCP) in HEPES (pH 7·5), followed by incubation and fluorescence measurement. Due to the ability of polymyxins to make a breach in the outer membrane lipopolysaccharides (LPS) for easy passage by other molecules, the ability of the capsules in keeping colistin away from breaching the LPS was confirmed (Campos *et al.* 2004) (Table 1).

Results showed that turbidity/optical density (representing cell viability) in the capsule-less 52K10 suspensions reduced substantially, compared to that of the capsule-forming 52145 suspensions. Also, NPN fluorescence was greatest in the 52K10 suspensions than that of the 52145 cell suspensions, suggesting that the capsules effectively inhibited the polymyxin B from reaching the LPS membranes to cause a disruption for the subsequent entry of the SDS, lysozyme and NPN. An intact LPS would have prevented the lysozyme, SDS and NPN probes from entry through the LPS. NPN is a hydrophobic molecule, which fluoresces when it interacts with hydrophobic molecules within the LPS and the CCCP was needed to prevent the extrusion of NPN by efflux pumps (Campos *et al.* 2004) (Table 1).

The level of capsule expression in 52145 was evaluated in the presence and absence of polymyxin B (after 3 h incubation) by measuring the uronic component of extracted capsules. Interestingly, there were more capsules in the cells incubated with polymyxin B than in those incubated without it. Subsequently, a transcriptional reporter assay was conducted by recombining the *cps* promoter cluster (responsible for the expression of the capsule genes) with the firefly luciferase gene *lucFF* (this gene encodes proteins that produces light in the firefly), using restriction enzymes and plasmid vectors. The *cps::lucFF* construct (that will result in light production anytime the capsule is expressed) was transformed into 52145 and the luminescence, during transcription, was measured in the presence and absence of the polymyxin with a luminometer. This corroborated the earlier finding that there is increased expression of capsules whenever capsule-forming *Kl. pneumoniae* cells are exposed to polymyxins (colistin) (Campos *et al.* 2004) (Table 1).

The lipid A moieties on the LPS of both 52K10 and 52145 cells were studied with MALDI-TOF MS and SDS-polyacrylamide gel electrophoresis (SDS-PAGE), but their peaks and patterns were the same, indicating that lipid A moieties could not be responsible for polymyxins resistance. Subsequently, a radial diffusion assay using *E. coli* C600 and 0·3 mg ml^{-1} tryptic soy broth (TSB) were undertaken. Supernatants obtained from centrifuging separately polymyxin (625 μl ml^{-1})-HEPES (pH 7·2) suspensions that had been incubated with purified capsules, LPS and live cells of 52K10 and 52145 were used to assay the amount of unbound polymyxin B in both strains as a measure of capsular ability in inhibiting polymyxin B binding to the LPS. There was no binding of the purified capsules to polymyxins (up to 400 mg ml^{-1}) while the 52K10 cells bound substantially more polymyxin (less polymyxin in the supernatant) than the 52145 cells; hence, capsules influenced the amount of polymyxin B reaching the LPS (Campos *et al.* 2004) (Table 1).

Lipid A mediates resistance to colistin: pmrHFIJFKLM mutation(s)

Yan *et al.* (2007) generated chromosomal deletions of *pmrL* and *pmrM* (genes that are part of the *pmrHFIJFKLM* operon (involved in lipid A biosynthetic pathway) in polymyxin-resistant *Salmonella Typhimurium MST100* and *E. coli* using in-frame kanamycin cassette replacements via restriction enzymes and plasmid vectors (Yan *et al.* 2007). Susceptibility was regained by the resistant isolates after the *pmrL-pmrM* deletions. Also, resistance was regained by the same *pmrL*-deletion mutants when they were transformed with full-length *pmrL* via pWSK29-*pmrL*. Subsequent electron-spray ionization quadrupole time-of-flight tandem mass spectrometer (ESI/MS) and thin-layer chromatography (TLC) assessment of the lipid A structure in the parental and mutant

Table 1 Colistin and tigecycline resistance mechanisms and their detection methods

Resistance mechanism	Detection method/tool	Parameter measured/used	Strain	Isolation year	Reference
Colistin					
Capsules	Spectrofluorometer	Fluorescence of NPN* Turbidity/optical density of capsular and noncapsular cells	*Klebsiella pneumoniae* 52145 (capsular serotype O1:K2), 52K10 (noncapsular), 52021 (LPS O antigen negative)	2004 2004	Campos *et al.* (2004)
	Transcriptional reporter assay (*cps:LucFF* construct)	Luminiscence (as a measure of transcription)			
	iELISA†	Capsules and LPS expression levels	*Kl. pneumoniae* 52145, 52ΔacrR		Padilla *et al.* (2010)
	Radial diffusion assay	Amount of bound/unbound polymyxins	Escherichia coli C600		Campos *et al.* (2004)
Lipid A modification	ESI/MS‡	L-Ara4N on lipid A	*Salmonella Typhimurium* MST100; *E. coli*	2007	Yan *et al.* (2007)
	TLC	pmrHFIJKLME/arnBCADTEF			
	Transcomplementation assay	colistin MICs in reference and transformed (Δ) strains			
Lipid A modification via phoPQ and pmrAB mutations	Transcomplementation assay	colistin MICs in reference and transformed (Δ) strains	*Pseudomonas aeruginosa* PA01	2009	Barrow and Kwon (2009)
	MALDI-TOF MS	L-Ara4N on lipid A	*Pseudomonas aeruginosa* PAK	2011	Miller *et al.* (2011)
	Transcriptional reporter assay (*pmrH:LacZ* construct)	phoP expression			
Lipid A modification via lpxA, lpxC and lpxD mutations	Whole genome sequencing (WGS) (Illumina)	SNPs in *pmrAB* and *lpxACD*	*Acinetobacter baumannii* ATCC 19606, 19606R	2010	Moffatt *et al.* (2010)
	Transcomplementation assay	colistin MICs in reference and transformed (Δ) strains			
	PAGE§	Presence/absence of lipid A			
	Carbohydrate –specific silver staining				
	Gas-Liquid Chromatography				
	CEEMS¶	Presence/absence of lipid A spectra			
	Limulus Amoebecyte Assay (LAL)	Presence/absence of lipid A			
acrAB efflux	Colony count	Survival rate of cells incubated with CCCP and with/out polymyxins	*Kl. pneumoniae* 52145, 52ΔacrB, 52ΔacrR	2004	Padilla *et al.* (2010)
ramA overexpression	Whole genome RNA sequencing (Illumina Hiseq)	Comparative transcriptome profiling	*Kl. pneumoniae* Ecl8, Ecl8ΔramA, Ecl8ΔramR	2015	De Majumdar *et al.* (2015)
	qRT-PCR**	ramA, OmpF, acrAB, lpxOLC expression levels			
	EMSA††	ramA binding site on genome			
	MALDI-TOF MS	Lipid A structural features/analysis			
	Antibiotic sensitivity tests	colistin MICs			
LPS	WGS	Sequence alignment and analysis	*Ac. baumannii* ColR, ColS	2015	Dafopoulou *et al.* (2016)

Table 1 (*Continued*)

Resistance mechanism	Detection method/tool	Parameter measured/used	Strain	Isolation year	Reference
Mcr-1	WGS	Presence of *mcr-1* gene	*E. coli* SHP45, C600, E11;	2013	Liu *et al.* (2015)
	Homology modelling	Structure of *mcr-1*	*Kl. pneumoniae* 1202, MPC11;		
	EIMS‡‡		*Ps. aeruginosa* HE26		
	Transformation assay	MICs of *mcr-1* transformants			
Tigecycline					
acrAB overexpression	Transcomplementation assay	colistin MICs in reference and transformed (Δ) strains	*Proteus mirabilis* G151, G6899ΔacrB, G6900ΔacrB	2003	Visalli *et al.* (2003)
			Morganella morganii G858, G1492, GC7676, GC7677, GC7743, GC7744	2005	Ruzin *et al.* (2005)
	Northern blot hybridization qRT-PCR	Expression levels of acrAB in parent and transformed (Δ) strains			
	Real-time qRT-PCR Western blotting	acrAB-tolC expression levels	*Kl. pneumoniae* (n = 57), *E. coli* (n = 19)	2007-2010	Roy *et al.* (2013b)
	E-test sensitivity testing with/out PAβN§§	MICs in the presence/absence of PaβN (efflux inhibitor)			
	Whole genome RNA sequencing (Illumina Hiseq)	Comparative transcriptome profiling: acrAB and ramA expression levels	*Kl. pneumoniae* Ecl8, Ecl8ΔramA, Ecl8ΔramR	2015	De Majumdar *et al.* (2015)
mexABCDXY-oprJM overexpression	Transcomplementation assay and broth micro-dilution	MICs in reference and transformed (Δ) strains	*Ps. aeruginosa* PA01(K767), PA01ΔmexB, PA01ΔmexXY, PA01ΔmexB/mexY, PA01ΔmexAB-oprM,	2003	Dean *et al.* (2003)
	GeneChip arrays	Tigecycline (TIG) effect on transcription levels			
	Western immunoblotting SDS-PAGE	oprJM transcription levels			
	Transcriptome profiling	Presence/absence of mutation in resistant strains			
16S rRNA binding site mutations	Transcomplementation assay and broth micro-dilution	MICs in reference and transformed (Δ) strains	*E. coli* TA527	2004	Bauer *et al.* (2004)
	rRNA methylation with dimethyl sulphoxide (DMS)	Binding site of TIG and binding site mutations			
IS5 insertion element and kpgABC efflux	WGS and sequences alignment	Mutations/Insertion sequences in resistant strains	*Kl. pneumoniae* KP40, KP47, KP49, KP52-1, KP64, KP66	2013/4	Nielsen *et al.* (2014)
	E-test- PaβN assay	MICs of cells treated/untreated with PaβN			
adeABC-FGH-IJK	RT-PCR	adeABC expression levels	*Ac. calcoaceticus-Ac. baumannii complex* G4906, G5139, G5140, G5141, GC7945, GC7951	2006	Ruzin *et al.* (2007)
			Clinical Ac. baumannii (n = 17)	2013	Yoon *et al.* (2013)
		adeFGH expression levels	*Clinical Ac. baumannii* (n = 14)	2010	Sun *et al.* (2010)
adeABC-FGH-IJK and *trm*	qRT-PCR	adeABC-FGH-IJK expression levels	*Ac. baumannii* 19606-T8, AT CC19606	2010	Chen *et al.* (2014)
	Transcomplementation assay	MICs of *trm* transformed strains			
Biofilms (Tigecycline and colistin)					

Table 1 (*Continued*)

Resistance mechanism	Detection method/tool	Parameter measured/used	Strain	Isolation year	Reference
Biofilm formation	Static biofilm assay Confocal scanning laser microscopy	Biofilm formation	*Kl. pneumoniae* ST258 (*n* = 28), ST327, ST376, ST277 and ST340	2013	Naparstek *et al.* (2014)
			Ac. baumannii (*n* = 3)	2015	Song *et al.* (2015)
	WGS trasncriptome profiling	Biofilm formation genes: *hha-ybaJ, ramA* overexpression	*Kl. pneumoniae* Ecl8, Ecl8ΔramA, Ecl8ΔramR	2015	De Majumdar *et al.* (2015)
	Static biofilm assays WGS	Biofilm formation Mutations/deletions in biofilm formation genes	*Ac. baumannii* clinical strains ColR and ColS	2015	Dafopoulou *et al.* (2016)

*1-naphthylamide (an efflux inhibitor).
†Inhibition enzyme-linked immunosorbent assay.
‡Electron-spray ionization quadrupole time-of-flight tandem mass spectrometer.
§Polyacrylamide gel electrophoresis.
¶Capillary electrophoresis electrospray mass spectrometry.
**Quantitative reverse-transcriptase PCR.
††Electrophoretic Gel Shift Mobility Assay.
‡‡Electrospray Ionization mass spectrometry.
§§Phe-Arg β-Naphthylamide.

strains showed that more than 95% of the *pmrL-pmrM* deletion mutants lacked L-Ara4N-modified lipid A, which was present in the resistant parental strains. These confirmed the involvement of the *pmrHFIJKLME* (also known as *arnBCADTEF*) operon in lipid A biosynthesis and subsequently, in polymyxin resistance (Yan *et al.* 2007) (Table 1).

phoPQ and *pmrAB* mediates resistance to colistin via regulation of lipid A biosynthesis

The role of the two-component regulatory genes *phoPQ* and *pmrAB*, which regulates the *pmrHFIJKLMpmrE* operon responsible for lipid A biosynthesis was ascertained using tetracycline and gentamycin gene cassettes to knock out a section of *oprH-phoPQ* (1870 bp), *pmrAB* (1466 bp) and *arnB* from the chromosomes of clinical *Ps. aeruginosa* PAO1 strains. These mutated genes were excised and transformed into polymyxin-resistant *Ps. aeruginosa* PAO1 strains to ascertain the effect of *pmrAB* and *phoPQ*, the two-component systems known to regulate *pmrHFIJKLM/arnBCADTEF*-mediated biosynthesis of lipid A, on polymyxin resistance. Resistant isolates transformed with full-length unmutated *pmrAB* and *phoPQ* genes reverted to lower MICs than that of the parent strain while strains transformed with mutated *pmrAB* and *phoPQ* were as resistant as the parent strain. On the contrary, Δ*arnB* transformants were susceptible to polymyxins, albeit their full-length unmutated *arnB* transformants were resistant. Comparative *pmrAB* and *phoPQ* sequence analysis of susceptible and resistant strains in a concurrent experiment showed identical mutations that were obviously responsible for the observed resistance. Thus, mutations in the two-component *pmrAB* and *phoPQ* systems were associated with polymyxin resistance while mutations in the *arnB* operon yielded a susceptible phenotype (Barrow and Kwon 2009) (Table 1).

In a similar experiment with *Ps. aeruginosa PAK* and their daughter cells transformed with knocked-out *pmrAB* and *phoPQ,* MALDI-TOF MS analysis of purified lipid A extracted from polymyxin sensitive and resistant isogenic strains showed consistent peaks that correlated with the presence of L-Ara4N modifications in the lipid A molecules of resistant phenotypes. Sequence analysis showed truncations, small and large in-frame deletions and frame-shifts in *pmrAB* and *phoPQ* as responsible for the observed resistance. Additional tests with a *lacZ* transcriptional reporter joined to a copy of the *pmrH* promoter region via restriction enzymes and plasmid vectors, indicated that *phoP* expression even in the absence of *phoQ* resulted in the addition of L-Ara4N to lipid A and subsequent polymyxin resistance (Miller *et al.* 2011) (Table 1).

Lipid A mediates resistance to colistin: lpxA, lpxC and *lpxD* mutation(s)

Moffatt *et al.* (2010) used a battery of tests to establish the effect of *lpxA, lpxC* and *lpxD* (genes that encode proteins that are involved in the biosynthesis of lipid A in *Ac. baumannii*) mutations on lipid A using a colistin resistant strain (19606R) of *Ac. baumannii* ATCC 19606 and its isogenic mutants. Whole genome sequencing of 19606R and sequences mapping with the parent 19606 strain showed no mutations in either *pmrA* or *pmrB* except a single nucleotide deletion (at position 90) that resulted in a premature termination of *lpxA* (at amino acid position 34)—*lpxA* encodes UDP-N-acetylglusamine, which catalyses the first step in lipid A biosynthesis. *LpxA*'s role in colistin resistance was further investigated by transforming 19606R with an intact *lpxA* gene. The MICs of the 19606RΔ*lpxA* reduced from 128 to 1 mg ml^{-1} (susceptible), indicating the role of *lpxA* in colistin resistance. PAGE and carbohydrate-specific silver staining of purified LPS and proteinase-K treated whole-cell lysates of the 19606, 19606R and 19606RΔ*lpxA* strains showed the absence of lipid A in only the 19606R strain, further confirming the role of *lpxA* in lipid A synthesis. Carbohydrate and fatty acid analysis of purified LPS with gas-liquid chromatography and capillary electrophoresis electrospray mass spectrometry showed consistent spectra and structural evidence of lipid A in 19606 and 19606RΔ*lpxA* but not in 19606R, corroborating the observation that lipid A was absent in the resistant strains (Moffatt *et al.* 2010) (Table 1).

Further analysis with limulus amoebocyte assay (LAL), which directly detects lipid A (endotoxin) in bacteria was carried out on the isolates. Lipid A was absent in the resistant 19606R. The hydrophobic NPN probe was used to evaluate the integrity of LPS in the resistant isolate by measuring the fluorescence emanating from the 19606, 19606R and 19606Δ*lpxA* cells. The 19606R strains emitted greater fluorescence than 19606 and 19606RΔ*lpxA* strains, suggesting that absence of lipid A in the LPS affected the membrane integrity of the resistant strains. This was further substantiated with antibiotic sensitivity studies. The colistin resistant strains were more sensitive to nonpolymyxin antibiotics than 19606 and 19606RΔ*lpxA,* due to the poor membrane integrity of the 19606R strains as a result of their loss of LPS (Moffatt *et al.* 2010) (Table 1).

AcrAB efflux pumps mediate resistance to colistin

Klebsiella pneumoniae 52145R (the rifampicin-resistant mutant of 52145) isogenic strains with *acrB* and *acrR* (which are part of the acrRAB efflux pump operon, respectively, encoding acrR, acrA and acrB; acrR regulates

the acrAB efflux system) knockouts (deficient) mutants, obtained through mutation-duplication mutagenesis assays, were used to evaluate their role in polymyxin resistance. In addition, inhibition enzyme-linked immunosorbent assay (iELISA) was used to evaluate the expression levels of LPS and capsules. The 52145R and 52Δ*acrR* strains expressed the same amounts of LPS and capsule. 52Δ*acrB* was most sensitive to polymyxin and several other antibiotics than 52145 and 52Δ*acrR*. Colony counts of polymyxin-exposed 52145R cells incubated with and without CCCP as well as CCCP and glucose were undertaken to assess the survival of the 52145 wild type cells in the absence of an active efflux. The survival of bacteria exposed to polymyxin and CCCP was expressed as a percentage of cells incubated without polymyxin. Cells incubated with CCCP-polymyxin had a greatly reduced survival rate compared with unexposed cells, but this disparity was reversed upon the addition of glucose to the polymyxin-CCCP solution for incubation, indicating that polymyxin resistance was influenced by an energy-dependent acrAB efflux pump activity (Padilla *et al.* 2010) (Table 1).

The ability of 52Δ*acrB* cells to cause pneumonia in murine models were carried out by infecting mice with 52145R and 52Δ*acrB cells* (Padilla *et al.* 2010). After 1, 2 and 3 days postinfection, the lungs of infected 52Δ*acrB*-mice had lesser bacterial loads ($P < 0{\cdot}05$), indicating that 52Δ*acrB* cells could not fully evade the host's cationic antimicrobial peptides (CAMP), which is similar in action to polymyxins. Hence, the importance of the acrAB efflux pumps in polymyxin resistance and virulence was established (Padilla *et al.* 2010).

RamA overexpression confers colistin resistance

RNA extracted from *Kl. pneumoniae* Ecl8 strain and its knockout mutants, *Ecl8ΔramA* and *Ecl8ΔramR,* were subjected to whole genome RNA sequencing, quantitative real-time reverse-transcriptase (qRT)-PCR and PCR to ascertain the effect of *ramA* expression levels, through comparative transcriptome profiling of the three strains (De Majumdar *et al.* 2015). Results showed that *ramA* overexpression in strain *Ecl8ΔramR,* led to OmpF (an outer membrane porin found in *Kl. pneumoniae*) down-regulation and upregulation of acrAB (effux pump), *lpxO, lpxL* and *lpxC* (genes important in lipid A biosynthesis). *In vitro* sequence analysis (IVT) and electrophoretic gel shift mobility assay (EMSA) indicated that purified *ramA* directly bound to and regulated *lpxO, lpxL, lpxC* (genes important in lipid A biosynthesis) and *acrAB* promoters, increasing their transcription (De Majumdar *et al.* 2015) (Table 1).

Subsequent MALDI-TOF analysis of the lipid A extracted from the strains showed lipid A structural modifications (observed as differences in peak patterns) in the r*amA*-overexpressing strain that were absent in the other strains, further substantiating that *ramA* affected colistin/polymyxin resistance levels by directly binding the lipid A biosynthesis genes *lpxO, lpxL and lpxC,* resulting in lipid A structural changes. Antibiotic sensitivity testing and survival assays also showed the *ramA* overexpressor as more resistant to all tested biocides (tetracyclines, aminoglycosides, fluoroquinolones, fungicides, toxic anions, macrolides and beta-lactams), including polymyxins. *RamA* thus affected polymyxin resistance through efflux and lipid A structural and regulatory genes (De Majumdar *et al.* 2015) (Table 1).

Mcr-1 confers resistance to colistin

Liu *et al.* (2015) recently identified a novel plasmid-borne PEt transferase gene, *mcr-1* that mediates colistin resistance in isolates from Chinese swine, raw pork and hospitalized patients. This gene transfers PEt to lipid A, reducing its anionic charge and subsequently, its affinity to the cationic colistin. The researchers suspected a plasmid-mediated colistin resistance mechanism by observing an increasing trend in colistin resistance among *E. coli,* which rarely undergoes chromosomal mutations to yield polymyxin resistance. The sequence and structure of the *mcr-1* gene and protein were, respectively, identified by WGS and homology modelling of the extracted plasmid pHNSHP45, using an IncI2 plasmid (pHN1122-1) as reference for the annotation. The *mcr-1* gene was located downstream an insertion sequence. The *mcr-1* gene and its flanks were ligated unto a pUC18 cloning vector and transformed into *E. coli* W3101 by electroporation. This transformant had a 4-fold MIC increase over the untransformed cells, indicating that *mcr-1* conferred resistance to colistin (Liu *et al.* 2015) (Table 1).

The researchers undertook conjugation and transformation experiments, respectively, between *E. coli* strains SHP45 (a porcine strain) and C600 and between *E. coli* SHP45 and *E. coli* E11, *Kl. pneumoniae* 1202 and MPC11, and *Ps. aeruginosa* HE26. These conjugants and transformants obtained 8-fold to 16-fold colistin MICs, confirming the transferability of *mcr-1* from SHP45 to C600 and E11 strains and to *Kl. pneumoniae* and *Ps. aeruginosa*. Serial passage tests with SHP45 and conjugated C600 strains in the presence and absence of colistin for 14 days, established the stability of the pHNSHP45 plasmid in both transconjugants and parent strains even in the absence of colistin selection pressure. *In vivo* resistance to colistin, under the mediation of the *mcr-1* gene was assessed with a murine thigh infection model using an *mcr-1* positive and *mcr-1* negative (cured) *E. coli* 363 strains. Over a 72-h period with 10^6 colony forming units of each strain and human colistin dosing, the carriage of

the cured (363S) strain reduced by more than three-log orders of magnitude compared to only one-log reduction in the *mcr-1* positive strain (363R). This suggested that the *mcr-1* offered protection (conferred resistance) to the *mcr-1* positive strain in the presence of colistin (Liu *et al.* 2015) (Table 1).

Tigecycline

Tigecycline is a novel 9-t-butyl glycylamido derivative of minocycline that was designed to maintain the broad spectrum antibacterial activity of tetracycline against both aerobic and anaerobic Gram-positive and Gram-negative bacteria (Tuckman *et al.* 2007; Kelesidis *et al.* 2008; Roy *et al.* 2013). Tetracycline resistance mechanisms of major clinical importance include the major facilitator superfamily (MFS) efflux pumps (*tetA, tetB, tetC, tetD* and *tetE*) and ribosomal protection proteins (*tetM*) (Petersen *et al.* 1999; Tuckman *et al.* 2007). Tigecycline is the first of a new class of antibiotics, the glycylcyclines and inspite of its broad antibacterial spectrum, *Ps. aeruginosa, Proteus spp., Providencia spp.* and *Morganella morganii* are known to be intrinsically less susceptible to it (Ruzin *et al.* 2005).

Resistance to tigecycline involves the upregulation of the acrAB-TolC OqxAB, and kpgABC efflux systems in Enterobacteriaceae under the regulation of *soxRS, robA, rarA, ramRAB* and *marRABC*. Mutations in these structural and regulatory genes confer resistance to tigecycline through upregulation of efflux pumps (Roy *et al.* 2013; Nielsen *et al.* 2014; De Majumdar *et al.* 2015).The MexABCDXY-oprJM efflux system is unique to *Ps. aeruginosa* and is regulated by the *mexR, mexZ* and *nfxb* regulatory genes that repress the overexpression of these efflux pumps, thus conferring susceptibility in wild type strains and resistance in strains bearing mutations in these structural and repressor genes (Dean *et al.* 2003). Similarly, the *adeABC-FGH-IJK* efflux pumps genes in *Ac. baumannii* are regulated by the *adeRSL* regulatory genes and mutations in these genes confer resistance to tigecycline through overexpression of efflux pumps (Ruzin *et al.* 2007; Chen *et al.* 2014).

*Hyper-transcription of acr*AB and *ram*A mediates resistance to tigecycline

The reason for the intrinsically low susceptibility of *Proteus mirabilis* to tigecycline was investigated with a clinically resistant *Pr. mirabilis G151* strain and its isogenic *acrAB* knockout mutants, G6899ΔacrB and G6900ΔacrB, in terms of their resistance to tigecycline. The knockout mutants, compared to the resistant parent strain, had 16-fold and 32-fold reduction in tigecycline and minocycline MICs respectively (using microbroth dilution, BMD). They also concurrently had a reduction in MIC to common acrAB substrates like novobiocin, chloramphenicol, fluoroquinolones, dyes and detergents but not to β-lactams that are not substrates of acrAB pumps. After transforming both the mutants and parent strain with a full-length acrAB gene via a plasmid vector, the MICs of the parent strain increased 4-fold and the mutants' MIC also increased 4-fold over the initial MIC of the parent strain. These results indicated the role of the acrAB efflux pump in tigecycline resistance. However, the *Pr. mirabilis* acrAB gene that was transformed into *E. coli* failed to increase its MIC, indicating the inability of acrAB-mediated tigecycline resistance to be spread across different genera (Table 1) (Visalli *et al.* 2003).

A similar study design was used to investigate the mechanisms underlying intrinsic tigecycline nonsusceptibility in *Morganella morganii* (Ruzin *et al.* 2005). Insertion of a transposon into the *acrA* gene of the resistant strain (resulting in a transformed mutant) resulted in a 128-fold reduction in MICs. Trans-complementation of the knockout mutant with a full-length *acrAB* gene restored tigecycline resistance and the multidrug resistance phenotype. Furthermore, Northern blot hybridization and quantitative real-time PCR (qRT-PCR) showed a 48·5-fold increase in *acrA* transcription level in the resistant strains than that of the susceptible ones (Ruzin *et al.* 2005). Roy *et al.* (2013) investigated mechanisms causing increased tigecycline resistance among *Kl. pneumoniae* clinical isolates obtained from neonates in a tertiary hospital in India. *E*-test strips of tigecycline and other antibiotics were used on Mueller-Hinton agar surfaces swabbed with Phe-Arg β-Naphthylamide (PaβN)-treated *Kl. pneumoniae* culture. The PaβN blocked efflux activity and resulted in a 2 to >8-fold reduction in the MIC of tigecycline as well as chloramphenicol, nalidixic acid, ciprofloxacin, tetracycline and minocycline (Roy *et al.* 2013) (Table 1).

Real-time RT-PCR of the *acrAB-TolC, soxS* and *ramA* genes identified the overexpression of *acrAB-TolC* (especially *acrA*) and *ramA* genes in both tigecycline resistant *Kl. pneumoniae* clinical isolates (Roy *et al.* 2013). *RamA* and *ramR* genes in susceptible and resistant *Kl. pneumoniae* clinical isolates were amplified by PCR and their sequences were compared, but no mutations were observed. Western blotting analysis of the *acrAB-TolC* genes, using polyclonal anti-*acrAB-TolC* antibodies confirmed the overexpression of *acrA* and *TolC* genes in the resistant isolates. The role of *ramA* in upregulating the *acrRAB* system has been further studied with whole genome transcriptome profiling of *Kl. pneumoniae* (described under colistin) (De Majumdar *et al.* 2015). These studies established the central role of the *acrAB* efflux system in tigecycline resistance.

mexABCDXY-oprJM overexpression confers resistance to tigecycline

Dean *et al.* (2003) used a laboratory *Ps. aeruginosa PAO1* (*K767*) with less susceptibility to tigecycline (MIC of 8 g ml^{-1}) and its spontaneously resistant knockout strains (PAO1ΔmexB, PAO1ΔmexXY, PAO1ΔmexB/mexXY and PAO1ΔmexAB-oprM) to study the mechanisms behind tigecycline resistance. MIC tests showed that the PAO1ΔmexXY and PAO1ΔmexAB-oprM strains were comparatively susceptible, suggesting that the mexXY and oprM efflux components are responsible for tigecycline resistance in the *Ps. aeruginosa PAO1* but not the mexAB system. The increased sensitivity of the PAO1ΔmexAB-oprM was due to the oprM (efflux component) as the PAO1ΔmexB strain had the same MIC as the parent strain PAO1. Through GeneChip arrays, the transcription effect of tigecycline on PAO1 cells exposed to tigecycline was assessed and it was established that different tigecycline concentrations (1–4 mg ml^{-1}) induced at least 12·8-fold increase in *mexXY* transcriptional levels. The *mexX* repressor gene, *mexZ*, was also expressed in the presence of tigecycline, possibly to serve as a negative feedback regulator to repress the overexpression of the mexXY pump (Table 1) (Dean *et al.* 2003).

Spontaneously resistant isolates obtained by exposing PAO1ΔmexXY, PAO1ΔmexB/mexXY and PAO1ΔmexAB-oprM isolates, that indirectly lack the mexXY and oprM efflux components, were further analysed with BMD, Western immunoblotting, SDS-PAGE and sequence analysis. The spontaneously resistant mutants were also multi drug resistant with MICs equal to that of the parent strain. These mutants overexpressed *mexAB-oprM* and *mexCD-oprJ*, showing that tigecycline is a substrate for these efflux pumps but only in the absence of a functional mexXY. In-frame deletions, genetic rearrangement and insertion sequence (IS) truncations were found in the *mexR* repressor gene located upstream the *mexAB-oprM* operon and in the *nfxb* (*mexCD-oprJ* repressor) gene located immediately upstream and in opposite orientation to the *mexCD-oprJ* gene, showing that these repressor genes were incapacitated through mutations to allow the overexpression of the *mexABCD-oprJM* genes (Table 1) (Dean *et al.* 2003).

Binding site mutations on 16S rRNA confers resistance to tigecycline

The mechanisms behind tigecycline resistance at the ribosomal level and the binding site of tigecycline on the 16S rRNA (70s ribosomes) were studied using dimethyl sulphate (DMS) modifications, MIC of mutated 16S rRNA *E. coli* transformants and Fe^{2+}-mediated hydroxyl radical cleavage. *E. coli* TA527 was transformed with plasmids bearing mutations in the *rrnC* operon, which encodes the 16S rRNA. Another *E. coli* strain, transformed with the *rrnB* operon (a component of the *rrnC operon* that encodes the 16S rRNA), was used as the wild type for the comparative MIC study. The MICs of the parent strain and mutants were determined using agar dilution methods and the *E. coli* TA527-transformed mutant was 8-fold more resistant to tigecycline than the *E. coli* strains transformed with the unmutated *rrnB* operon. rRNA methylation with DMS was tested in the presence of tigecycline and tetracycline as well as rRNA modification sites were identified by primer extension, which was analysed (electrophoresed) with denaturing polyacrylamide sequencing gels (Table 1) (Bauer *et al.* 2004).

Phosphoimager analysis identified the presence and quantity of the modifications while the positions of the modifications were mapped by dideoxynucleotide sequencing. In addition, Fe^{2+}-mediated and Mg^{2+} competition of Fe^{2+}-mediated hydroxyl radical cleavage of tigecycline and tetracycline bound to the 70S ribosome, followed by primer extension analysis of the rRNA as described above, showed that tigecycline, as well as tetracycline, bound to the same sites on the rRNA by protecting the binding site from DM methylation; this was identified through the presence of three cleavage sites regions on the rRNA. Therefore, mutations in the *rrnBC* operon encoding the rRNA influenced tigecycline susceptibility (Table 1) (Bauer *et al.* 2004).

kpgABC efflux mediates resistance to tigecycline

A new RND efflux system, named *kpgABC,* has been implicated in tigecycline resistance that evolved/emerged in a *Kl. pneumoniae* during therapy in a 2·5-month-old Honduran neonate using comparative whole genome analysis. The MICs of susceptible and resistant strains were determined using *E*-test strips on PAβN-treated Mueller-Hinton agar. MICs of the resistant strain reduced 4-fold. Comparative mapping of the susceptible and resistant isolates' whole genome sequences, showed no mutations in *rarA, marA, ramA, ramR, acrA, Omp-like* and *oqxA* genes, but an IS*5* insertion site with the target repeat sequences CTAAGTG and CTAAGG. Furthermore, an inverted repeat sequence AAGGTGCGAAYAAG was found within a promoter region close to the *kpgABC* operon conserved in some *Kl. pneumoniae* isolates. Quantitative reverse trancriptase PCR (qRT-PCRA) analysis showed increased *kpgA* (132-fold) and *kpgB* (48-fold) expressions in the resistant isolate. Transformation of the *kpgABC* operon into a susceptible *Kl. pneumoniae* strain resulted in a 4-fold MIC increase. This new efflux pump (*kpgABC*) is thus an additional mechanism conferring resistance to tigecycline (Table 1) (Nielsen *et al.* 2014).

adeABC-FGH-IJK and *Trm* mediates resistance to tigecycline

Ruzin *et al.* (2007) studied the mechanisms involved in tigecycline resistance in four isogenic *Acinetobacter calcoaceticus-Ac. baumannii* complex strains obtained from a single patient in the US and their *adeB* knockout mutants. Real-time RT-PCR indicated the overexpression of the *adeA* gene (and by extension, the *adeBC* genes as they are co-transcribed) 27–37-fold in nonsusceptible isolates while MIC studies with *adeB* knockout mutants revealed a great reduction in tigecycline MIC. By comparing the sequences of the *adeRS* locus, which regulates *adeABC,* the authors found IS*Aba-1* disrupting the *adeS* genes in the resistant strains. However, this was not seen in another study (Ruzin *et al.* 2007; Yoon *et al.* 2013) where 17 unrelated clinical *Ac. baumannii* $bla_{OXA\text{-}48\text{-}like}$ carbapenemase-producing isolates, seven of which were resistant to tigecycline, were studied; *E*-test with and without an efflux pump inhibitor (NMP) spread onto a Mueller-Hinton agar and RT-PCR of the *adeB* gene was used to describe tigecycline resistance mechanisms in these strains from Taiwan. The tigecycline MICs in the resistant isolates, which overexpressed the *adeB* efflux component 57·6 times over the susceptible isolates, reduced substantially in the presence of the NMP efflux inhibitor (Table 1) (Sun *et al.* 2010).

qRT-PCR and conventional PCR were used to analyse 14 nonclonally related tigecycline resistant clinical *Ac. baumannii* isolates expressing $bla_{OXA\text{-}48\text{-like}}$ and $bla_{GES\text{-}5}$ carbapenemases in France. *AdeB* was found in 13 isolates and its expression was high (>20-fold) among most of the tigecycline resistant isolates ($n = 10$), *adeG* was found in all isolates and overexpressed (4–15-fold) in eight isolates, but *adeJ* was not overexpressed although it was present in all isolates. Through sequence analysis and homology modelling of the *adeRS* and *adeL* regulatory genes, the authors found no mutations in the *adeL* gene but observed important functional mutations in *adeS* (R152K, N125K, G336S, H189Y and I252S) and *adeR* (P56S, L129R and E219A) that were implicated in tigecycline resistance (Table 1) (Yoon *et al.* 2013).

Chen *et al.* (2014) selected a tigecycline resistant *Ac. baumannii* laboratory strain (19606-T8) obtained through serial passage experiments from an ATCC 19606 parent strain and used WGS, comparative genome-wide analysis and trans-complementation tests to investigate the mechanisms underlying the evolution of tigecycline resistance in the 19606-T8 strain. Expression analysis with real-time qRT-PCR of the *adeABC, adeFGH* and *adeIJK* genes revealed no substantial overexpression and could therefore not be responsible for the observed resistant phenotype. Comparative genome-wide analysis of the 19606-T8 and ATCC19606 strains, corroborated by conventional PCR and Sanger sequencing, revealed four mutations in the genome of the 19606-T8 strain (*trm*, *msbA, lolA* and *filC* genes) but only two genes (*trm* and *filC*) correlated with increased tigecycline resistance. Wild type *trm, msbA, lolA* and *filC* cloned into plasmid vectors were transformed into the resistant 19606-T8 strain but only the *trm* transformant reversed tigecycline's MIC from 8 to 1 mg l^{-1} and also reversed resistance to tetracycline, doxycycline and minocycline in the same manner. *Trm* was identified as a constitutive gene in *Ac. baumannii* encoding the S-adenosyl-L-methionine-dependent methyltransferase. The S-adenosyl-L-methionine mutation, resulting in a frameshift and truncated protein in the 19606-T8 strain, mediated resistance to tigecycline and other tetracyclines (Table 1) (Chen *et al.* 2014).

Biofilms formation as a resistance mechanism (tigecycline and colistin)

Biofilms are known to play an important role in bacterial infections, but their direct role in tigecycline and colistin resistant carbapenemase producers is less described. Pathogenic bacteria are known to reside in matrix-enclosed biofilms within surfaces inside the body and on invasive devices, making them inaccessible to antibiotics. The natural tendency of pathogens to associate in biofilms, which facilitate easy horizontal transfer of resistance genes between members, instead of existing as free-moving individual planktonic cells, is well documented (Naparstek *et al.* 2014; Song *et al.* 2015).

The effect of colistin on biofilm formation and the effect of biofilms in mediating resistance to colistin were studied with 46 clinical *Kl. pneumoniae* isolates that were resistant to colistin in Tel-Aviv, Israel. Most of the isolates ($n = 28$) were of ST258 although other sequence types ($n = 18$) were also identified, viz., ST327; ST376; ST277; ST340. Static biofilm assays in polystyrene microtitre 96-well plates was used to measure biofilm formation using crystal violet staining and subsequent quantification of the optical density (OD_{590}). These were repeated in the absence and presence of increasing concentrations of colistin. Confocal scanning laser microscopy was further used to analyse the 3D structure and quantify the volumes of the formed biofilms using image and biofilm analysis software. While colistin did not increase biofilm formation, *Kl. pneumoniae* isolates within biofilms were 2–3-fold more resistant than planktonic cells, suggesting that biofilms played a role in colistin resistance but its formation was not induced by the presence of colistin (Table 1) (Naparstek *et al.* 2014).

The biofilm formation properties of three clinical carbapenemase-producing *Ac. baumannii* isolates, grown in

the presence and absence of tigecycline, colistin, colistin-rifampicin, imipenem-rifampicin and rifampicin using the polystyrene micro-titre plate assay, were evaluated. After crystal violet staining, optical densities of the positive and negative control *Ac. baumannii* cultures were measured with a spectrophotometer. Colistin was not effective against *Ac. baumannii* (in biofilms) at normal MIC levels but tigecycline was very effective in inhibiting biofilm formation even at one-fourth its MIC levels. Subsequently, biofilms are effective mechanisms in colistin resistance in *Ac. baumannii* but not a tigecycline resistance mechanism (Song *et al.* 2015). As described under the experimental basis of colistin resistance, comparative expression analysis using *Kl. pneumoniae Ecl8ΔramR, Ecl8ΔramA* and *Ecl8* strains demonstrated the direct effect of *ramA* on the bio-film forming genes, *hha-ybaJ*. *RamA* was shown to directly bind to these biofilm forming genes (*hha-ybaJ*), leading to their overexpression and subsequent biofilm formation. Hence, *ramA* induces increased biofilms formation, and subsequently increases colistin resistance (De Majumdar *et al.* 2015). Alignment of the whole genome sequences of colistin susceptible (ColS) and resistant (ColR) *Ac. baumannii* clinical strains showed deletions of biofilm forming genes and mutations in *lpsB*, involved in LPS synthesis; hence, biofilm formation was deficient in ColR (Table 1) (Dafopoulou *et al.* 2016).

Methods and challenges in detecting tigecycline and colistin resistance

The main methods used to detect colistin and tigecycline resistant Gram-negative bacteria can be classified under phenotypic and genotypic methods. Phenotypic methods include culture-based disc diffusion or micro-broth dilution (BMD) antibiotic sensitivity testing in the presence and absence of efflux pump inhibitors, spectrophotometry, MALDI-TOF MS and immunological assays (such as iELISA and LAL). However, spectrophotometry, MALDI-TOF MS and immunological/biochemical assays are rarely used. These phenotypic tests require prior 24-h incubation to obtain fresh bacterial cultures. Genotypic methods used in detecting colistin and tigecycline resistant bacteria include conventional PCR, real-time PCR, qRT-PCR and WGS. These genotypic tests rely on the detection of mutations in the structural and regulatory genes influencing lipid A and efflux pumps expression and/or an evaluation of the comparative expression levels of the structural genes (Chen *et al.* 2014; Olaitan *et al.* 2014; De Majumdar *et al.* 2015). Hence, the molecular tests require advanced skill and require an additional turnaround time beyond the 24-h incubation time required for the simpler phenotypic tests.

The resistance mechanisms in Gram-negative bacteria (cell wall and outer membrane/LPS—lipid A in colistin resistance and efflux pumps in tigecycline resistance—and their regulation) pose a difficulty in designing a detection method for tigecycline and colistin resistance with <24 h turnaround time (Beceiro *et al.* 2014; Nielsen *et al.* 2014; De Majumdar *et al.* 2015). Except for the recently detected *mcr-1* gene, colistin and tigecycline resistance mechanisms differ from that of other antibiotics, in which resistance is mainly mediated by a drug-modifying enzyme as is the case for carbapenemases (Osei Sekyere *et al.* 2015; Sekyere *et al.* 2016). Invariably, a faster and cheaper colistin and tigecycline resistance detection method might depend on colistin and tigecycline concentrations and not on modifying enzymes, except for the *mcr-1* gene. With the emergence of *mcr-1* colistin resistance gene in Gram-negative bacteria in swine, raw meat and hospitalized patients in China and Malaysia (Liu *et al.* 2015), a faster detection method is needed to quickly identify and contain colistin and tigecycline resistant bacteria from spreading, thus saving these two antibiotics of last resort.

Phenotypic methods (colistin and tigecycline)

Susceptibility testing breakpoints

The mainstay of colistin and tigecycline phenotypic resistance detection in the clinical microbiology and research laboratories continues to be culture-based disc diffusion (Kirby Bauer's method) with colistin and tigecycline impregnated-discs, E-test or BMD. The BMD is thus far the phenotypic gold standard for colistin and tigecycline sensitivity testing. Moreover, colistin and tigecycline breakpoints for most Gram-negative species are recommended only for BMD. The Food and Drug Administration (FDA) and EUCAST tigecycline breakpoints are commonly used as there is no breakpoint for tigecycline by CLSI for all Gram-negative bacteria. However, CLSI has colistin breakpoint for only *Ps. aeruginosa* and recommends 10 μg discs (disc diffusion), while EUCAST recommends 30 μg colistin discs (disc diffusion) for Enterobacteriaceae and *Ac. baumannii* (Table 2) (Clinical and Laboratory Standards Institute (CLSI), 2015; European Committee on Antimicrobial Susceptibility Testing, 2015).

EUCAST has no tigecycline breakpoints for *Ps. aeruginosa* and *Ac. baumannii* and no colistin and tigecycline breakpoints for *Stenotrophomonas maltophilia*. EUCAST recommends a different MIC breakpoint ($S \leq 1$ mg l^{-1}, $R > 2$ mg l^{-1}) from that recommended by the FDA ($S \leq 2$ mg l^{-1}, $R > 8$ mg l^{-1}) (Cohen Stuart *et al.* 2010; Zarkotou *et al.* 2012 European Committee on Antimicrobial Susceptibility Testing, 2015). Occasionally, the BSAC

Table 2 Tigecycline and colistin breakpoints recommended by CLSI, EUCAST, FDA and BSAC

Bacterial Family/ Species	Interpretative breakpoint standard	Colistin		Tigecycline		References
		Disc diffusion (mm)	Micro-broth dilution (mg l^{-1})	Disc diffusion (mm)	Micro-broth dilution (mg l^{-1})	
Enterobacteriaceae	CLSI	–	–	–	–	Clinical and Laboratory Standards Institute (CLSI), (2015)
	EUCAST	S ≥ 11, R ≤ 10 (30 μg disc)	S ≤ 2, R > 2	S ≥ 18, R < 15	S ≤ 1, R > 2	European Committee on Antimicrobial Susceptibility Testing, (2015)
	FDA	–	–	–	S ≤ 2, R ≥ 8	Brust *et al.* (2014); Kanj *et al.* (2014)
	BSAC	–	–	S ≥ 20, R < 20	S ≤ 1, R > 2	Casal *et al.* (2009); Hope *et al.* (2010)
Pseudomonas aeruginosa	CLSI	S ≥ 11, R ≤ 10 (10 μg)	S ≤ 2, R ≥ 8	–	–	Clinical and Laboratory Standards Institute (CLSI), (2015)
	EUCAST	–	S ≤ 4, R > 4	–	–	European Committee on Antimicrobial Susceptibility Testing, (2015)
	FDA	–	–	–	S ≤ 2, R ≥ 8	Brust *et al.* (2014); Kanj *et al.* (2014)
	BSAC	–	–	–	–	Casal *et al.* (2009); Hope *et al.* (2010)
Acinetobacter baumannii	CLSI	–	S ≤ 2, R ≥ 4	–	–	Clinical and Laboratory Standards Institute (CLSI), (2015)
	EUCAST	–	S ≤ 2, R > 2	–	–	European Committee on Antimicrobial Susceptibility Testing, (2015)
	FDA	–	–	–	S ≤ 2, R ≥ 8	Brust *et al.* (2014); Kanj *et al.* (2014)
	BSAC	–	–	S ≥ 20, R < 20	S ≤ 1, R > 2	Casal *et al.* (2009); Hope *et al.* (2010)

breakpoints, which agrees with EUCAST, are employed for tigecycline (S ≤ 1 mg l^{-1}, R > 2 mg l^{-1}) (Table 2) (Casal *et al.* 2009; Hope *et al.* 2010).

Evaluation studies on tigecycline susceptibility testing

Several multi-centre studies have evaluated the detection tests—disc diffusion, Vitek 2, *E*-test, agar dilution, MIC test strip and micro-broth dilution—used in tigecycline and polymyxin antibiotic sensitivity testing. These analyses were done on a broad range of Gram-positive and Gram-negative bacteria using the micro-broth dilution and/or agar dilution as the reference method. The discrepancies in MICs of the Vitek 2 system were found to be higher in 241 carbapenem-resistant Enterobacteriaceae (CRE) and *Ac. baumannii* and 48 KPC-producing *Kl. pneumoniae* compared to the E-test results. However, another study reported otherwise, with 49/94 *E*-test discrepancies as against 38/73 Vitek 2 discrepancies (Lat *et al.* 2011; Zarkotou *et al.* 2012). Polymyxin B *E*-test has showed an error a rate of 10/19 in 48 KPC-producing *Kl. pneumoniae* (Lat *et al.* 2011). Several multi-centre evaluation studies involving Enterobacteriaceae and *Ac. baumannii* have confirmed that tigecycline *E*-test results are always several dilutions higher than that recorded by BMD, except at low MIC levels (0·5–1 mg l^{-1}) (Casal *et al.* 2009), and that tigecycline *E*-test nonsusceptible results need to be confirmed with micro-broth dilution to avoid false resistant results (Pillar *et al.* 2008; Casal *et al.* 2009; Cohen Stuart *et al.* 2010).

Available breakpoints for tigecycline disc diffusion have shown an overestimation of sensitivity (Jones *et al.* 2007; Kulah *et al.* 2009; Hope *et al.* 2010), which reduces drastically upon carefully reducing/adjusting the breakpoints (Kulah *et al.* 2009; Hope *et al.* 2010). However, it has been argued in an evaluation study on Enterobacteriaceae ($n = 6$) involving several culture media, that the media used in tigecycline sensitivity testing also affects the outcome, irrespective of the methods used (Cohen Stuart *et al.* 2010; Torrico *et al.* 2010). Hence, micro-broth dilution must be used in colistin and tigecycline sensitivity testing whenever possible with a standardized media. The phenotypic methods are cheap and easy to use but have a longer turnaround time of 24-h.

Molecular/Genotypic Methods (tigecycline and colistin)

Conventional PCR, real-time qRT-PCR and WGS are commonly used, mainly by reference laboratories to understand the resistance mechanisms underlying the observed phenotypic resistance to tigecycline and colistin. Conventional PCR is used to amplify known genes mediating resistance to these two antibiotics: *mgrB, phoP, phoQ, pmrAB, pmrHFIJKLM/arnBCADT, acrRAB-TolC, adeABC, mexXY-oprM, mexAB-oprM* (Roy *et al.* 2013;

Beceiro *et al.* 2014; Nielsen *et al.* 2014; De Majumdar *et al.* 2015). The PCR amplicons are sequenced and analysed by comparing these sequence to the same genes in wild type or susceptible strains for a possible mutation in the amplified gene (Nielsen *et al.* 2014; De Majumdar *et al.* 2015). In addition, qRT-PCR is also employed to study the expression levels of these genes in both resistant and susceptible or wild type strains to ascertain the transcription rate of the respective resistance genes in the resistant phenotype (Roy *et al.* 2013; Nielsen *et al.* 2014; De Majumdar *et al.* 2015). To understand the genetic determinants of resistance to tigecycline and colistin beyond the confines of the identified genes, WGS of the bacterial genome and/or RNA is normally employed, followed by comparative mapping or alignment to a wild type or susceptible reference strain's genome or RNA (Nielsen *et al.* 2014; De Majumdar *et al.* 2015). New resistance determinants have thus been identified (Chen *et al.* 2014; Nielsen *et al.* 2014).

These methods require skills and expensive reagents that are not common in many clinical microbiology laboratories in developing countries. Moreover, the initial culture step required to undertake molecular analysis increases the turnaround time of this approach although it is good for epidemiological and research purposes. These methods are faster than the phenotypic tests without an initial culturing step. Evaluation studies comparing the different methods for colistin and tigecycline molecular detection are needed.

Potential detection methods for future research and development

For a faster detection of colistin resistance in Gram-negative bacteria, there is potential in MALDI-TOF MS as a proteomic tool in detecting the presence, absence and modification of the lipid A, capsule and LPS structure which are integral to colistin resistance (Campos *et al.* 2004; Moffatt *et al.* 2010; Miller *et al.* 2011). Immunological tools like LAL that directly detects lipid A as an endotoxin could also be further developed to facilitate a faster and cheaper detection of colistin resistant Gram-negative bacteria (Moffatt *et al.* 2010). Because tigecycline resistance is largely attributable to efflux pumps and/or 16S rRNA (70S ribosomes) mutations, directly determining tigecycline resistance will be difficult (Yim *et al.* 2011; Roy *et al.* 2013; Nielsen *et al.* 2014). However, because these two resistance mechanisms result in an unchanged tigecycline concentration and structure in the extracellular (and/or intracellular) matrix, concentration and structure-based measurements with spectrophotometric and MALDI-TOF MS tools may be potential methodologies that could be used to measure changes in free/unbound tigecycline concentration as opposed to ribosome-bound tigecycline concentration to identify characteristics unique to resistant strains. Finally, WGS and real-time multiplex PCR and/or micro-array technology can be further developed with primers and probes targeting the *mcr* gene such that variants of this gene can be identified in a single reaction; novel colistin and tigecycline resistance genes can be easily detected via WGS (Chen *et al.* 2014; Nielsen *et al.* 2014; De Majumdar *et al.* 2015).

Conclusion

With the first report of plasmid-borne colistin resistance (*mcr-1*) and the increasing dependence on tigecycline and colistin for the treatment of fatal infections, colistin and tigecycline resistance is expected to escalate among carbapenem-resistant Gram-negative bacteria through mutations in efflux and lipid A structural and regulatory genes respectively. There is thus the need to develop faster detection tools for easy identification, infection control and containment. Improved and faster detection methods should focus on the presence or modifications in *mcr-1*, lipid A, LPS and/or capsules and on the structure-concentration relationship of free and bound extracellular tigecycline. Also, methods that can quickly detect the ratio of viable: nonviable bacterial concentrations in a standardized colistin or tigecycline solutions/suspensions over a time period would be pertinent.

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Conflict of Interest

No conflict of interest declared.

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