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Rapid high-resolution detection of colistin resistance in Gram-negative bacteria using flow cytometry: a comparison with broth microdilution, a commercial screening test and WGS

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Background: Even though both EUCAST and CLSI consider broth microdilution (BMD) as the reference method for antimicrobial susceptibility testing (AST) of colistin, the method exhibits potential flaws related to properties of the colistin molecule.

Objectives: To develop a flow cytometry method (FCM) for colistin AST and to validate it against BMD, a commercial screening test and WGS.

Methods: Colistin-mediated loss of membrane integrity in *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter* spp. was detected with the fluorescent probe YoPro-1 by FCM. An international collection of 65 resistant and 109 susceptible isolates were analysed and the colistin concentration required to reach the EC_{50} was compared with the BMD MIC and the presence of genotypic resistance markers.

Results: The overall FCM sensitivity and specificity for colistin resistance was 89% and 94%, with *E. coli* > *K. pneumoniae* > *P. aeruginosa*, whereas the performance for *Acinetobacter* spp. was poor. All tested *E. coli* were correctly categorized. Three *K. pneumoniae* isolates with genotypic findings consistent with colistin resistance were detected by FCM but not BMD. Compared with BMD, FCM delivered AST results with a 75% reduction of time.

Conclusions: Here, we present a rapid FCM-based AST assay for qualitative and quantitative testing of colistin resistance in *E. coli* and *K. pneumoniae*. The assay revealed probable chromosomal colistin resistance in *K. pneumoniae* that was not detected by BMD. If confirmed, these results question the reliability of BMD for colistin testing.

Introduction

Due to the emergence of carbapenemase-producing Enterobacterales and MDR *Pseudomonas aeruginosa* and *Acinetobacter* spp., colistin is again a part of the therapeutic arsenal for the treatment of severe bacterial infections. However, its role is under debate due to ambiguities regarding its effectiveness.[1] Furthermore, the appearance of alterations in chromosomal and/or transferable genes conferring colistin resistance has made rapid and reliable antimicrobial susceptibility testing (AST) for colistin challenging.[2] Disc diffusion tests are widely recognized as inadequate, while gradient strips have been shown to be unreliable to identify low-level resistance.[3] Both EUCAST and CLSI consider broth microdilution (BMD) as the reference method, while CLSI also accepts two alternative methods (disc broth elution and colistin agar test).[4]

Despite this recommendation, the reliability of BMD for colistin testing has been questioned. Due to the amphipathic nature of colistin, the drug is positively charged at physiological pH, which enables the primary interaction with negatively charged lipid A in the LPS of the outer membrane of Gram-negative bacteria.[5] However, the cationic molecule also adsorbs to commonly used laboratory

plastics, thus affecting the concentration of available colistin in the broth,[6,7] resulting in a risk of overestimating the true MIC. Upon binding to target bacteria, colistin causes membrane disruption and subsequent cell death.[5] The action of colistin is considered rapid; however, substantial regrowth over time has been noted in time–kill experiments.[8,9] Due to colistin's prompt action, assays with a short exposure time could potentially be plausible alternatives for susceptibility testing. Several methods producing either qualitative AST results or MICs have been proposed,[10–12] and recently promising results for MIC determination using a flow cytometry method (FCM) have been reported, although as a patented assay with few technical details revealed.[13]

Phenotypic AST of bacteria is, by tradition, based on $\log_2$ dilutions of antimicrobial agents. When validating new AST methods, BMD MICs are considered as a reference by both EUCAST and CLSI. According to published criteria, novel methods should achieve at least 90% MIC agreement within ±1 dilution step against BMD (essential agreement, EA).[14] However, by nature, BMD MICs are discrete data, meaning that the actual concentration needed to inhibit growth of a bacterial isolate could be somewhere between the measured MIC and the preceding $\log_2$ concentration. A different way of measuring potencies of drugs is through regression analysis, using response as a function of drug concentration. This approach utilizes the effective dose to reach half-maximum response (EC_{50}) and is widely used in general pharmacology but has only occasionally been described for AST.[15,16] In contrast to BMD MICs, EC_{50} values represent continuous data from which statistical analysis can be performed, including determination of CIs. If the EC_{50} approach was proven feasible for AST, these CIs could be used to define WTs, ECOFFs and for setting clinical FCM breakpoints for different drug/bug combinations.

Here, we aimed to develop and explore an FCM-based assay for colistin AST in *Escherichia coli, Klebsiella pneumoniae, P. aeruginosa* and *Acinetobacter* spp., based on the detection of compromised membrane integrity in cells and EC_{50} modelling of FCM data. The evaluation was performed using a diverse bacterial strain collection, including strains with various degrees and mechanisms of colistin resistance. The performance was extensively evaluated, both as a screening tool for colistin resistance (qualitative AST) and as a method for MIC prediction (quantitative AST) using BMD as a primary comparator. Existing MIC breakpoints for BMD were used as tentative breakpoints also for FCM. In addition, the assay performance was compared with that of a commercially available rapid test. In-depth analysis of genotype/phenotype correlations was performed using WGS, focusing on key genes associated with colistin resistance (*mcr, mgrB, phoPQ, pmrAB* and *crrAB*).[17]

Materials and methods

When not stated otherwise, chemicals, reagents and laboratory utensils were procured from Thermo Fisher Scientific. Bacteria were cultured onto blood (Merck KGaA) or Uri*Select*™ 4 (Bio-Rad) agar, and CAMHB (Becton, Dickinson and Company) was used in all suspension cultures except for BMD. Sterile filtration was performed using Nalgene filter units (PES 0.1 μm, VWR International LLC).

Strain collection

Bacterial isolates (*E. coli, K. pneumoniae, P. aeruginosa* and *Acinetobacter* spp.) of local (Department of Clinical Microbiology, Region Kronoberg, Sweden) and international [kindly provided by the EUCAST development laboratory (EDL)] origin were selected based on previous colistin AST results. The aim was to compose a strain collection with at least 25% resistant strains and 25% with MICs within ±1 dilution step from the clinical breakpoint. Moreover, a minimum of 20 isolates/species was sought. The final collection contained 97 local and 77 international isolates; the species distribution is described in Table 1. All strains were subjected to colistin AST as described below. WGS was performed on a subset of *E. coli* ($n = 28$) and *K. pneumoniae* ($n = 28$), including all isolates with discrepant results from BMD/FCM testing.

MALDI-TOF MS (Bruker Daltonic GmbH) was used for species identification before preserving all of the strains in glycerol stock at −70°C. All experiments were performed using fresh, overnight-cultured colonies.

BMD MIC

BMD was performed with the Sensititre™ microtitre system using custom-made FRCOL plates, containing lyophilized colistin sulphate, resulting in final concentrations of 0.125–128 mg/L according to the manufacturer's instructions [1×10^5 cfu/mL in CAMHB with TES, 50 μL/well]. MIC was determined as the lowest concentration with absence of visual growth after 16–20 h of incubation at 35°C. Testing and reading were performed by staff with extensive experience of BMD on clinical samples, in a laboratory with rigorous external quality assessment and frequent use of recommended quality control strains.

Flow cytometry AST

The protocol for FCM AST, including the choice of YoPro-1 as a marker for membrane-compromised cells, was established after optimization, briefly described in the Supplementary methods and Figures S2 and S3, available as Supplementary data at *JAC* Online. Bacterial colonies were suspended to 0.5 McFarland in 0.9% NaCl and inoculated (100 μL) in 11 mL sterile filtered CAMHB in glass tubes. The tubes were incubated for 90–100 min at 35°C, after which broth was transferred to Sensititre™ plates (100 μL/well) using an AIM Sensititre™ dispensing robot. Three types of Sensititre™ plates with varying resulting colistin concentration ranges were used: FRCOL 0.06–64 mg/L; SEMPA1 0.25–32 mg/L; and SEMEN6 0.25–32 mg/L (the latter two kindly provided by the EDL, Växjö, Sweden). The quality of all plates was ensured by regular testing using recommended strains.[18] The plates were sealed with adhesive cover tape and incubated [30 min, 35°C, 450 rpm (Thermal Shake Lite, VWR)]. FCM was performed after addition of 25 μL/well of YoPro-1 (25 μM in Hanks balanced salt solution). Collected data were subsequently analysed using FlowJo™ software, version 10.5.0 (Becton, Dickinson and Company). Identical trimming procedures were sequentially applied on all FCM data. The bacterial cell population was identified and gated by forward scatter (FSC) and side scatter (SSC) parameters (Figure 1a), followed by exclusion of doublets by FSC^{area} versus FSC^{height} analysis (Figure 1b). For each isolate, baseline fluorescence on BL1 (YoPro-1) was identified in unexposed wells and a corresponding YoPro-1-positive/negative separator gate was set and applied on all colistin concentrations (Figure 1c).

Flow cytometry settings

FCM was performed on the Attune™ NxT system, including autosampler and software (v.2.5), with Attune™ Focusing Fluid as sheath fluid. The flow rate was set to 25 μL/min with data acquisition starting after 3 s of aspiration, and when possible proceeding to a minimum of 20 000 events. Before each aspiration, six rinsing cycles and three mixing cycles were performed. The laser was set to 488 nm and data were collected from FSC, SSC and BL1 (530/30 nm) channels. The voltage/threshold settings (FSC 300 V/200, SSC 320 V/200 and BL1 260 V) were identical for analysis of *E. coli, K. pneumoniae* and *Acinetobacter* spp. For *P. aeruginosa*, FSC 480 V/100, SSC

Table 1. Characteristics of bacterial strains included in the study

	Number of local isolates		Number of international isolates		
Species	S (WGS)	R (WGS)	S (WGS)	R (WGS)	Sum (WGS)
E. coli	34 (14)	5 (0)	5 (5)	9 (9)	53 (28)
K. pneumoniae	26 (10)	13 (0)	6 (6)	12 (12)	57 (28)
P. aeruginosa	8 (0)	5 (0)	12 (0)	9 (0)	34 (0)
Acinetobacter spp.[a]	6 (0)	—	12 (0)	12 (0)	30 (0)
All isolates	74 (24)	23 (0)	35 (11)	42 (21)	174 (56)

S, susceptible isolates (MIC $\leq$ 2 mg/L); R, resistant isolates (MIC > 2 mg/L).
[a]Including 18 *A. baumannii*, 1 *Acinetobacter bereziniae*, 2 *Acinetobacter haemolyticus*, 1 *Acinetobacter junii*, 3 *Acinetobacter lwoffii*, 4 *Acinetobacter pittii* and 1 *Acinetobacter seifertii*.

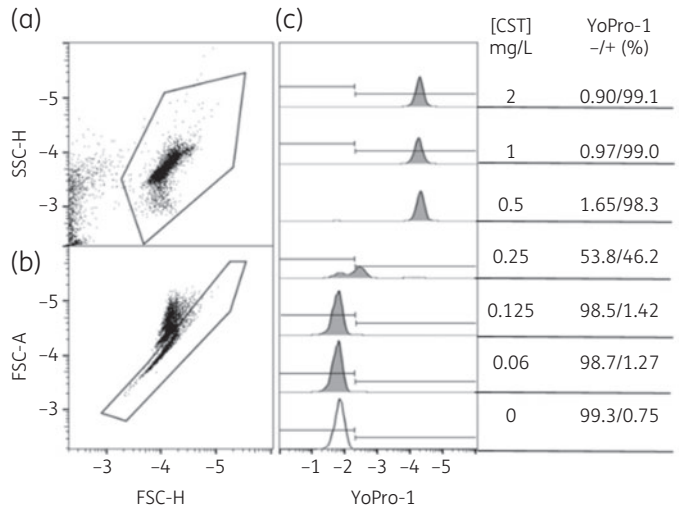

Figure 1. FCM analysis. All events plotted on FSC-H (log_{10}) versus SSC-H (log_{10}) and gated to distinguish noise signal from bacterial cells (a). The bacterial cell population was selected and doublet removal was performed (b). The single cell population was selected and a histogram was created. The growth control wells were used to set YoPro-1 gates that were applied for all colistin concentrations (c). Colistin (CST) concentration and the corresponding fractions of YoPro-1-positive and -negative cells are depicted in the columns to the right.

320 V/100 and BL1 260 V settings were applied. Before FCM, all fluids were sterile filtered.

Dose–response modelling

The YoPro-1-positive fraction of cells (%) in each well was plotted against the log_{10} of the corresponding colistin concentration and regression analysis was performed [*log(agonist) vs. response—Variable slope* (four parameters), Graph Pad Prism v.7.04 for Windows, GraphPad Software]; see Equation 1 below [Y, response (% YoPro-1-positive cells); X, log colistin concentration (mg/L); Bottom; plateau (% YoPro-1-positive cells); Top, plateau (% YoPro-1-positive cells); Hill slope, slope factor; EC_{50} in mg/L]. The EC_{50} was calculated for each dataset. Where applicable, standard deviation (SD) was used as a measure of spread and significance testing was performed using unpaired *t*-test (GraphPad Prism). For the qualitative analysis, $EC_{50} \leq 2$ mg/mL was considered susceptible and analytical sensitivity and specificity for colistin resistance (including 95% CI) were calculated using BMD as reference (Graph Pad Prism, Wilson–Brown method). For MIC comparison against BMD, the estimated EC_{50} of the tested strain was rounded up to the closest log_2 MIC step and designated EC_{50}^{MIC}.

$$Y = Bottom + (Top - Bottom)/(1 + 10((logEC_{50} - X) \times Hill\ slope)) \quad (1)$$

The FCM assay's robustness was evaluated through repeated analysis of control strains recommended for colistin testing: *E. coli* ATCC 25922 and *E. coli* NCTC 13846.[18]

Rapid Polymyxin NP™

Qualitative detection of colistin resistance was performed through analysis with Rapid Polymyxin NP™ (*E. coli, K. pneumoniae*), Rapid Polymyxin™ Pseudomonas (*P. aeruginosa*) or Rapid Polymyxin™ Acinetobacter (*Acinetobacter* spp.) (ELITech Group, Puteaux, France) according to the manufacturer's instructions. Analytical sensitivity and specificity were calculated as described above, using the readout at the longest recommended incubation time according to each product.

Genotypic characterization using next-generation sequencing

WGS, followed by *de novo* assembly and genomic characterization of species, MLST, capsule loci and ESBL- and carbapenemase-encoding genes, was performed as described in the Supplementary methods. The raw reads are available under BioProject PRJEB35248 and sample accession numbers and genotyping results are listed in Table S1.

To identify genetic characteristics associated with colistin resistance, the presence of *mcr* genes, non-synonymous single nucleotide variations (SNVs) and ISs in *mgrB, phoP, phoQ, pmrA, pmrB, crrA* and *crrB* were analysed. PROVEAN v1.1.3 was used to predict the functional impact of the SNVs on the protein sequence.[19]

Results

The strain collection used was highly heterogeneous with regard to phenotypic susceptibility, with MICs ranging from 0.25 to 128 mg/L, as determined by BMD. When including all four species, the proportion of resistant isolates was 37%, and 65 of 174 isolates had MICs within ±1 dilution step from the susceptible/resistant breakpoint for colistin (2 mg/L).

Table 2. Qualitative performance of FCM

Species	FCM						Rapid Polymyxin NP™					
	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	CA (%)	ME (%)	VME (%)	*n*	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	CA (%)	ME (%)	VME (%)	*n*
E. coli	100 (78–100)	100 (91–100)	100	0	0	53	100 (78–100)	100 (91–100)	100	0	0	53
K. pneumoniae	100 (87–100)	91 (76–97)	95	5	0	57	100 (86–100)	94 (7–97)	96	4	0	55[a]
P. aeruginosa	79 (52–92)	90 (70–97)	85	6	9	34	100 (70–100)	15 (5–36)	41	59	0	29
Acinetobacter spp.	67 (39–86)	89 (67–98)	83	7	13	30	100 (72–100)	68 (42–85)	81	19	0	26[a]
All species	89 (80–95)	94 (87–97)	90	4	4	174	100 (94–100)	77 (69–84)	85	15	0	163
All species except *Acinetobacter* spp.	94 (85–98)	95 (88–98)	93	3	2	144	100 (92–100)	76 (66–83)	85	15	0	130

Analytical sensitivity, specificity, CA, MEs (false resistance) and VMEs (false susceptibility) of FCM AST and Rapid Polymyxin NP™ in comparison with the BMD method. EC_{50} susceptible classification cut off: ≤2 mg/L.
[a]Two *K. pneumoniae* and four *Acinetobacter* isolates were omitted from analysis due to control growth failure.

Flow cytometry for detection of colistin resistance

In the total collection of strains ($n = 174$), FCM exhibited an overall sensitivity and specificity for detecting colistin resistance of 89% and 94%, respectively (Table 2). The best performance was seen in *E. coli* and *K. pneumoniae*, with 100% sensitivity and >90% specificity in both species, very similar to the performance of the Rapid Polymyxin NP™ test. Both major errors (MEs, false resistance) and very major errors (VMEs, false susceptibility) were observed at a rate of 4% in the entire tested collection; however, most of these occurred in *P. aeruginosa* and *Acinetobacter* spp. Excluding these species eliminated all VMEs and after this only three isolates with MEs remained for *K. pneumoniae* (described below). Analysing the mean FCM-derived EC_{50}, BMD susceptible strains significantly differed from the resistant population in all species, with $P < 0.0001$ for *E. coli* and *K. pneumoniae*, $P = 0.001$ for *P. aeruginosa* and $P = 0.0121$ for *Acinetobacter* spp. (Figure 2). Three BMD susceptible *K. pneumoniae* presented as outliers, with $EC_{50} > 2$ mg/L, and are described in detail below. When excluding *Acinetobacter* spp. from the analysis, the sensitivity and specificity for colistin resistance increased to 94% and 95%, respectively. Due to the FCM's lower sensitivity for colistin resistance in *P. aeruginosa* and *Acinetobacter* spp., these two species were omitted from further analysis.

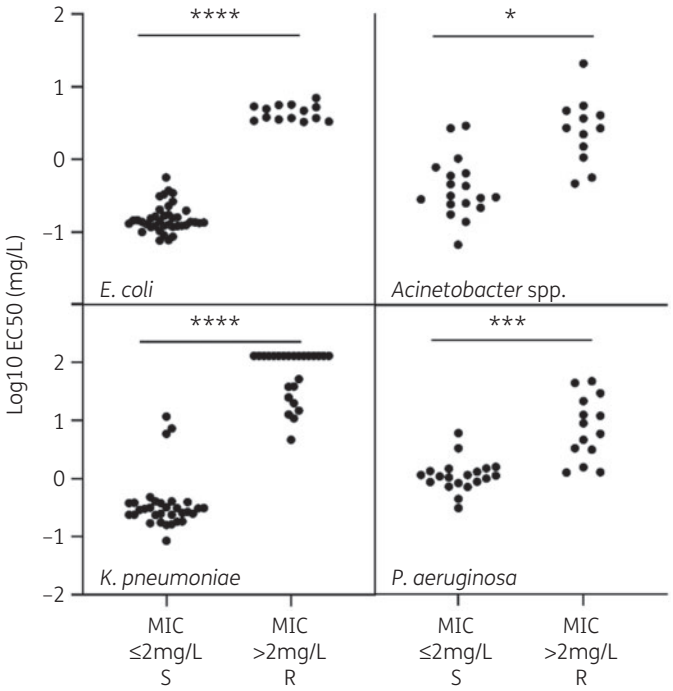

Figure 2. Estimated EC_{50} for 53 *E. coli*, 57 *K. pneumoniae*, 34 *P. aeruginosa* and 30 *Acinetobacter* spp. after non-linear regression analysis of FCM data. EC_{50} out of range (>64 mg/L) was plotted as 128 mg/L and omitted from significance testing. Isolates with colistin MIC ≤ 2 mg/L were considered susceptible (S) and >2 mg/L resistant (R), as determined by BMD. Each strain was analysed one to five times. When repeated, the mean EC_{50} is reported. The statistical significance of differences (unpaired *t*-test) is indicated by horizontal lines; ****, $P < 0.0001$; ***, $P = 0.001$; *, $P < 0.05$.

Flow cytometry AST and MIC prediction

All international isolates of *E. coli* and *K. pneumoniae* and a subset of the local isolates of the same species (*E. coli*, $n = 28$; *K. pneumoniae*, $n = 28$) were subject to further investigation. The proportion of colistin resistance remained unchanged; however, the subset included all isolates with discrepant results between BMD and FCM. Upon exposure to increasing colistin concentrations (0.06–64 mg/L), FCM analysis revealed a dose-dependent increase of the fraction of YoPro-1-positive cells. The YoPro-1 signal, as a marker of membrane-compromised cells, presented with a steep shift from a primarily YoPro-1-negative cells to a majority of YoPro-1-positive cells over one to two concentration steps (Figures 1c and 3). Plotting the fraction of YoPro-1-positive cells against the $\log_{10}$ colistin concentration resulted in sigmoidal shaped curves, which for most susceptible strains reached nearly 100% positive cells at the highest concentrations (Figure 3). However, particularly in some high-grade resistant *K. pneumoniae* isolates, the curves did not reach a plateau and EC_{50} was considered out of range for these isolates (>64 mg/L).

When comparing EC50MIC with BMD MIC (Figure 4a), the categorical agreement (CA) was 100% for *E. coli* and 90% for *K. pneumoniae*, while the EA only reached 79% and 52%, respectively. Calculation of EC50MIC deviation from BMD MIC showed that the median EC50MIC was one dilution step lower than MIC (*E. coli*,

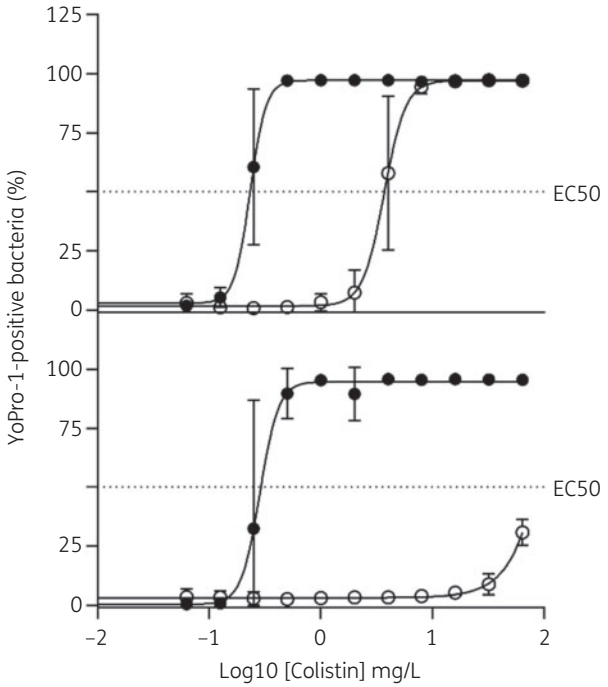

Figure 3. Representative examples of non-linear regression analysis of the percentage of YoPro-1-positive cells as a function of colistin concentration (log_{10}) on susceptible (filled circles) and resistant (open circles) *E. coli* (top) and *K. pneumoniae* (bottom). The error bars denote the SD of the percentage of positive cells from at least three repeated individual experiments.

$P = 0.0032$; *K. pneumoniae*, $P = 0.0039$) in susceptible isolates. In resistant strains, the opposite was found; *E. coli* isolates differed with +1 ($P = 0.0313$) and *K. pneumoniae* +3 ($P = 0.0005$) dilution steps (Figure 4b).

Flow cytometry AST in accordance with genotypic colistin resistance markers

Nine *E. coli* and the only *K. pneumoniae* isolate carrying *mcr* were identified as resistant by FCM with EC50MIC of 4–8 mg/L (*E. coli*) and 16 mg/L (*K. pneumoniae*). Additionally, one susceptible *E. coli* (COL-17) carried *mcr-1*, but with a frameshift likely rendering it non-functional. In total, 9 of 12 *K. pneumoniae* isolates containing alterations in or loss of *mgrB* were detected as phenotypically resistant by both BMD and FCM. A summary of the detected *mgrB* alterations is shown in Figure S1. Both methods classified one isolate (COL-72) with an *mgrB* truncation due to an ISKpn26-like element as susceptible.

For the remaining two isolates with *mgrB* truncations (COL-15 and COL-74), FCM but not BMD indicated potential resistance. One additional isolate (COL-94) was classified as resistant by FCM while susceptible with BMD, without any detected changes in *mgrB*. Instead, the strain exhibited non-synonymous SNVs in *pmrA* (G53S) and *crrB* (G114R), both predicted by PROVEAN as having deleterious effects on protein function. A summary of phenotypic and genotypic findings in all *K. pneumoniae* isolates with reduced colistin susceptibility according to any of the employed methods is shown in Table 3.

All three discrepant *K. pneumoniae* isolates had colistin BMD MICs at the high end of the WT range (1 mg/L), and their corresponding EC50MICs were 8 mg/L (COL-74, COL-94) and 16 mg/L (COL-15). Their dose–response curves differed from other WT isolates, having an attenuated response to increasing concentrations of colistin, including a flatter slope of their respective dose–response curves (Hill slope 1.7–2.8; WT median 4.4) (Figure 4c).

A high degree of reproducibility and accuracy of EC_{50} in quality control strains

On repeated testing, *E. coli* ATCC 25922, with MIC target 0.5–1 mg/L and QC acceptance range of 0.25–2 mg/L, presented with mean EC_{50} of 0.17 ± 0.05 mg/L, corresponding to EC50MIC of 0.25 mg/L ($n = 15$). For the *mcr-1*-positive control strain *E. coli* NCTC 13846, mean EC_{50} was 1.89 ± 0.66 mg/L ($n = 15$), corresponding to EC50MIC of 2 ($n = 10$) or 4 mg/L ($n = 5$), in full accordance with the QC acceptance range of 2–8 mg/L. The intra/inter assay coefficients of variation (CV) of the obtained EC_{50} values were calculated as 18%/31% and 33%/37% for ATCC 25922 and NCTC 13846, respectively (Figure 5).

Discussion

We have developed a novel FCM assay for the rapid detection of colistin-mediated resistance in clinically relevant Gram-negative bacteria. The method has been validated against a well-characterized collection of strains, with highly varying degrees of colistin susceptibility, a broad range of MLST and capsule types (*K. pneumoniae*) and a wide diversity of resistance mechanisms to colistin. Great effort was made to ensure that the strain collection was challenging, with many strains having an MIC close to the clinical colistin breakpoint. Additionally, the method was highly reproducible and less variable than the BMD method.

The cellular effects of colistin exposure were detected using the fluorescent probe YoPro-1, which is well documented as a live/dead stain in mammalian cells.[20,21] However, to our knowledge, this is the first time it has been utilized for AST of bacteria. From bacterial colonies, total time to results using our protocol is less than 3 h for one isolate. Adding an autosampler streamlines the assay, enabling parallel testing of multiple strains. Colistin concentration ranges can be chosen to cover either an entire MIC distribution or be narrowed down to breakpoint concentrations, depending on the clinical needs.

As a screening tool for colistin resistance, FCM performed well compared with the commercially available Rapid Polymyxin NP™ tests for *E. coli* and *K. pneumoniae*.[10,11] With sensitivity and specificity of 79% and 90%, the performance of FCM was not as convincing in *P. aeruginosa*; however, the results are comparable with the manufacturer's performance specifications for the Rapid Polymyxin NP™ Pseudomonas test. Even though our FCM assay appears insufficient as colistin AST for *P. aeruginosa* in clinical practice, in our hands it was far more specific than the Rapid Polymyxin NP™ Pseudomonas, which erroneously classified 17 of 20 susceptible strains as resistant, using BMD results as the reference. Our experience of the Rapid Polymyxin NP™ Pseudomonas test stands in stark contrast to previously published data, which suggested a specificity of 95%. However, in that paper all 40 susceptible strains had MICs of <0.5 mg/L.[12] In the current evaluation, 8 of 9 susceptible *P. aeruginosa* had MIC 1 mg/L, corresponding to the mode WT MIC, and several challenging isolates were included. FCM exhibited a low sensitivity for the detection of colistin resistance in

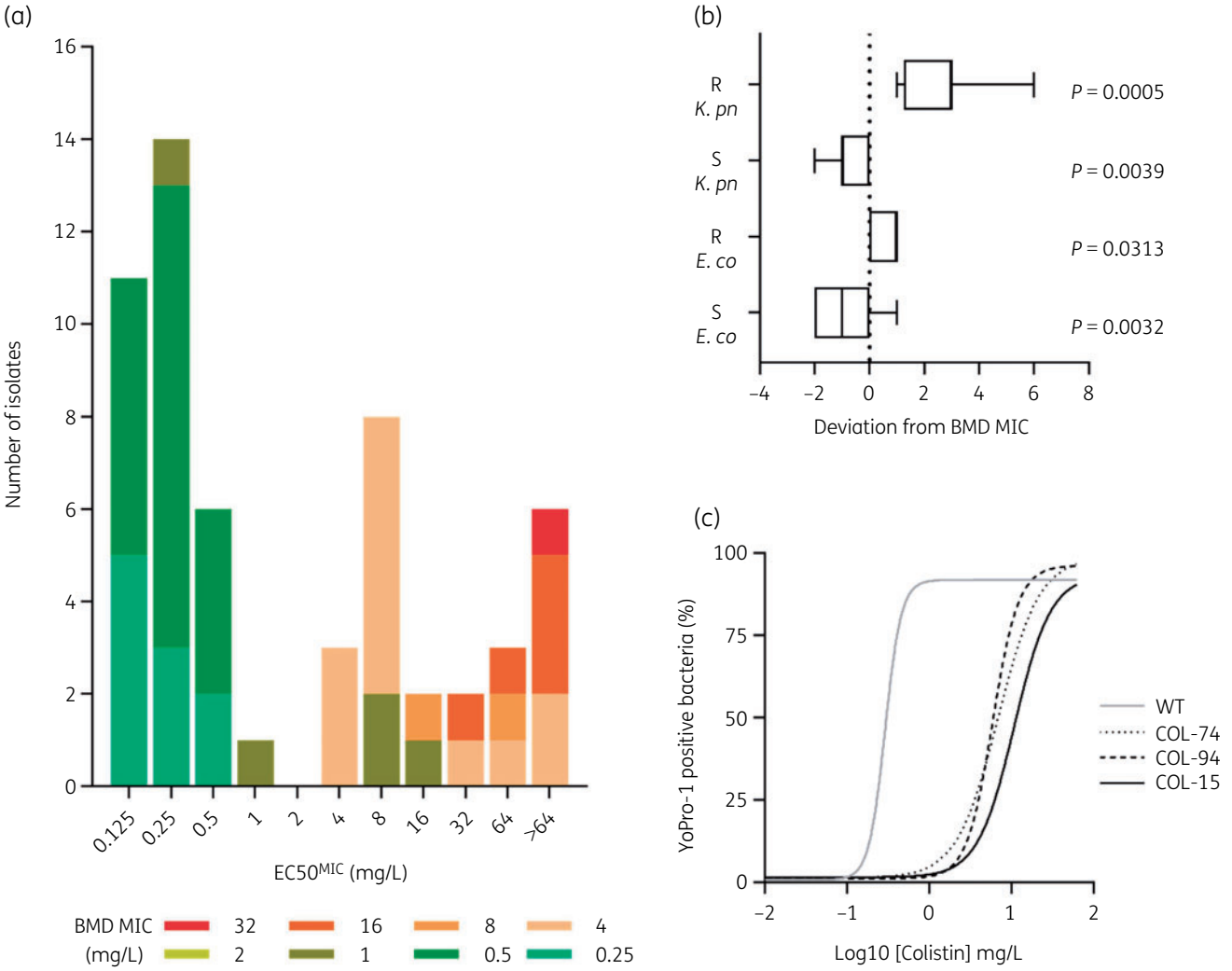

Figure 4. (a) Frequency histogram of estimated EC50MIC in 28 *E. coli* and 28 *K. pneumoniae* isolates analysed with FCM (bars) and colour coded by their respective BMD-derived MIC. (b) Significant (Wilcoxon signed rank test) deviation of estimated EC50MIC from BMD-derived MIC, expressed as concentration steps (log_2) above (positive) and below (negative) target MIC (dotted line) in 19 susceptible and 9 resistant *E. coli* and 13 susceptible and 12 resistant *K. pneumoniae*. Three discrepant isolates were omitted from the analysis; see (c). Boxes, 25%–75% quartiles; line, median deviation; whiskers, minimum to maximum range. E. co, *E. coli*; K. pn, *K. pneumoniae*; S, susceptible; R, resistant; p, two-tailed *P* value, Pratt method. (c) Dose–response, non-linear regression curves of three discrepant *K. pneumoniae*, COL-15 ($n = 3$), COL-74 ($n = 3$) and COL-94 ($n = 1$), and four different WT *K. pneumoniae* strains ($n = 12$).

Acinetobacter spp. The reason for this is not clear, but initial response to low concentrations of colistin followed by regrowth at 24 h has previously been reported in colistin-resistant strains of *Acinetobacter baumannii*.[22] The clinical relevance of this phenomenon remains to be explored.

Several new methods for detection of *mcr*-mediated resistance have been suggested. However, since these are usually based on detecting the gene or the gene product, they can at best predict resistance but never guarantee susceptibility.[23,24] Even though the FCM assay is not exclusively specific for plasmid-mediated colistin resistance, it exhibited excellent ability to identify low-grade resistant isolates carrying *mcr*. All resistant *mcr*-positive *E. coli* had MICs of 4 mg/L, while their corresponding EC_{50}s ranged from 3.3 to 5.7 mg/L. For the only *K. pneumoniae* with confirmed *mcr-1*, the EC_{50} was 10.9 mg/L and its corresponding MIC 8 mg/L.

In *K. pneumoniae*, resistance to colistin is commonly mediated through chromosomal changes in *mgrB*, eventually leading to lipid A modifications. The FCM assay was able to identify all resistant isolates with confirmed *mgrB* truncations, as well as two isolates classified as susceptible by BMD. One additional BMD susceptible isolate was identified as resistant by FCM. WGS of these discrepant isolates showed genetic changes fully compatible with colistin resistance in all three strains. Their BMD MICs were all at the high end of the WT range (1 mg/L), two of the three isolates were also detected as resistant using the Rapid Polymyxin NP™ test and all three grew after 48 h of incubation on Superpolymyxin™ agar, a commercially available agar plate for the screening of colistin resistance in Gram-negatives (ELITech MICROBIO) (data not shown).[25] Although preliminary, these data indicate that in certain circumstances, FCM may be superior to BMD in identifying chromosomal resistance to colistin.

Table 3. Summary of genetic and phenotypic characteristics

Isolate	MLST	*wzi* gene	K locus	ESBL CPE	BMD		FCM		RP	*mcr mgrB*	Non-synonymous variants or truncations		
					MIC (mg/L)	S/R	EC_{50} (mg/L)	S/R	S/R		*phoP*/*phoQ*	*pmrA*/*pmrB*	*crrA*/*crrB*
COL-2	147	wzi64	KL64	KPC-2	4	R	50.7	R	R	Premature stop codon *mgrB* (L4*)	*phoQ* (D150G)	**_pmrB_ (R256G)**	
COL-3	45	wzi121	KL43	CTX-M-15 OXA-48	16	R	>64	R	R	IS element insertion in *mgrB*	*phoQ* (D150G)	**_pmrB_ (R256G**)	*crrB* (Q287K)
COL-5	39	wzi83	KL23	KPC-2	4	R	>64	R	R	IS element insertion in *mgrB*	*phoQ* (D150G)	*pmrA* (M66I)	
COL-15[a]	147	wzi64	KL64	VIM-27	1	S	11.7	R	S	IS element insertion in *mgrB*	*phoQ* (D150G) **_phoQ_ (L199Q)**	**_pmrB_ (R256G)**	
COL-19	377	wzi173	KL102	CTX-M-15	8	R	10.9	R	R	*mcr-1*	*phoQ* (D150G)	*pmrB* (V247I)	*crrB* (Q296L)
COL-32	101	wzi137	KL17	CTX-M-15 OXA-48	16	R	>64	R	R	Premature stop codon *mgrB* (Q30*)	*phoQ* (D150G)	**_pmrA_ (D86E)** *pmrA* (A217V)	
COL-33	147	wzi64	KL64	CTX-M-15 NDM-1	16	R	24.6	R	R	IS element insertion in *mgrB*	*phoQ* (D150G)	**_pmrB_ (R256G**)	
COL-34	147	wzi64	KL64	CTX-M-15	32	R	>64	R	R		**_phoQ_ (R16S)** *phoQ* (D150G) **_phoQ_ (R187L)**	**_pmrB_ (R256G)**	
COL-35	101	wzi137	KL17	CTX-M-15 OXA-48	16	R	>64	R	R	*mgrB* (M27K)	*phoQ* (D150G)	*pmrA* (A217V)	
COL-36	101	wzi137	KL17	KPC-3	4	R	>64	R	R	*mgrB* gene missing	*phoQ* (D150G)	*pmrA* (A217V)	
COL-37	101	wzi137	KL17	CTX-M-15 OXA-48	16	R	37.5	R	R	*mgrB* (M27K)	*phoQ* (D150G)	*pmrA* (A217V)	
COL-38	15	wzi24	KL24	CTX-M-15 OXA-48	8	R	37.9	R	R	IS element insertion in *mgrB*	*phoQ* (D150G)	*pmrB* (A246T)	
COL-71	525	wzi45	KL45	CTX-M-15 NDM-1 OXA-181	4	R	19.9	R	R		*phoQ* (D150G)	**_pmrA_ (G53C)** **_pmrA_ (D86E)** *pmrB* (G345E)	crrA (I219V)
COL-74[a]	147	wzi64	KL64	VIM-27	1	S	7.3	R	R	IS element insertion in *mgrB*	*phoQ* (D150G) **_phoQ_ (L199Q)**	**_pmrB_ (R256G)**	
COL-94[a]	ST759-2LV	wzi349	KL147		1	S	5.9	R	R		*phoQ (D150G)*	**_pmrA_ (G53S)** *pmrA* (E57G)	***crrB* (G114R)**

Data from WGS *K. pneumoniae* isolates that tested resistant by any of the methods BMD, FCM or Rapid Polymyxin NP™ (RP).
[a]Isolates with divergent results. Genetic variants of *phoP*/*phoQ*, *pmrA*/*pmrB* and *crrA*/*crrB* predicted as neutral by PROVEAN with regard to protein function are shown in normal text and those predicted deleterious in bold text. CPE, carbapenemase; S, susceptible; R, resistant.

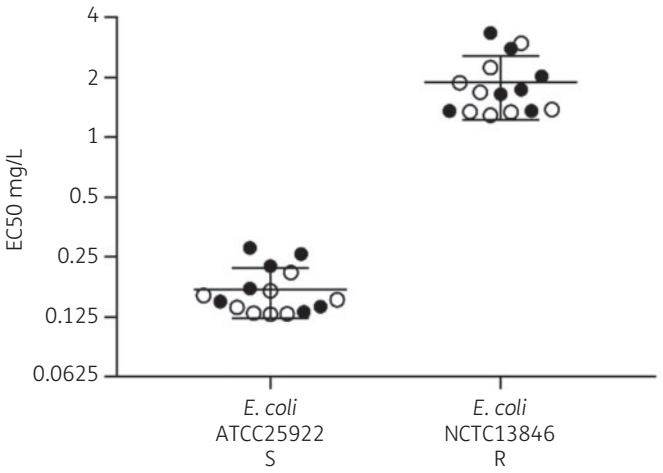

Figure 5. Estimated EC_{50} values (mg/L) from repeated FCM analysis of two recommended quality control *E. coli* strains. Filled circles, inter assay; open circles, intra assay; horizontal line, mean of all repetitions; error bars, SD; S, susceptible, R; resistant.

One *K. pneumoniae* isolate (COL-72) with an *mgrB* truncation due to an ISKpn26-like element was classified as susceptible by both BMD and FCM. While the IS and insertion site in this isolate are identical to what previously has been described in colistin-resistant strains, this isolate also had an SNV leading to an N253T amino acid substitution in *phoQ*, which has been hypothesized to render *mgrB*-disrupted strains susceptible to colistin.[26,27]

FCM-based AST methods have been described previously, most recently by Fonseca and colleagues, who presented a patented method for colistin AST.[13,28] In contrast to that assay, our method utilizes standard laboratory equipment and widely available reagents. Even though this evaluation was performed using an acoustic flow cytometer, the assay could theoretically be run on other flow cytometers with sufficient resolution for small particle detection.[29–34] However, the performance of the assay needs to be evaluated on each platform before considering it for use on clinical samples. Although the previously described method seems to produce a higher degree of essential MIC agreement, a direct comparison is difficult due to the absence of strain-specific data in the published papers (e.g. species-specific MIC data and genotypic data other than *mcr*). Moreover, in a recent publication by Castanheira *et al.*,[35] BMD retesting of 200 *K. pneumoniae* isolates resulted in EA of only 60%, indicating reproducibility issues also with the refence method.

The present study has limitations: firstly, WGS was not performed on all isolates; secondly, the assay performance with respect to resistance mechanisms other than lipid A modification has not been addressed; thirdly, the lower EC50MIC for susceptible and higher EC50MIC for resistant isolates needs to be further explored by timescale experiments; and fourthly, BMD and FCM were not always performed on the same subculture. However, we believe that the last limitation has had only a minor impact, if any, on the results and that such an impact would rather have led to an underestimation of the performance of FCM-based MIC prediction than the opposite.

In conclusion, we propose an FCM-based colistin AST assay validated for qualitative screening of colistin resistance in *E. coli* and *K. pneumoniae*. The assay additionally allows for quantification of the degree of resistance (EC50MIC). The assay revealed possible resistance in three *K. pneumoniae* isolates genetically compatible with acquired colistin resistance that were not detected by the BMD method, indicating that BMD in its current form is not optimal for colistin resistance detection and that there may be room for other, faster and equally or more accurate methods such as FCM.

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Transparency declarations

Thermo Fisher Scientific Inc. provided reagents for flow cytometry. The company did not take part in experimental design, data handling, result analysis or conclusions, and has not influenced the outcome of the work.

Supplementary data

Supplementary methods, results and references, Table S1 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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