Clinical Microbiology and Infection xxx (2017) 1–8

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

Automated time-lapse microscopy a novel method for screening of antibiotic combination effects against multidrug-resistant Gram-negative bacteria

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ARTICLE INFO

Article history:
Received 13 July 2017
Received in revised form
28 September 2017
Accepted 31 October 2017
Available online xxx

Editor: W Couet

Keywords:
Antibiotic combinations
Carbapenemases
Escherichia coli
Image analysis
Klebsiella pneumoniae
oCelloScope
Synergy
Time–kill experiments

ABSTRACT

Objectives: Antibiotic combinations are often used for carbapenemase-producing *Enterobacteriaceae* (CPE) but more data are needed on the optimal selection of drugs. This study aimed to evaluate the feasibility of a novel automated method based on time-lapse microscopy (the oCelloScope, Philips BioCell A/S, Allerød, Denmark) to determine *in vitro* combination effects against CPE and to discuss advantages and limitations of the oCelloScope in relation to standard methods.

Methods: Four *Klebsiella pneumoniae* and two *Escherichia coli* were exposed to colistin, meropenem, rifampin and tigecycline, alone and in combination. In the oCelloScope experiments, a background corrected absorption (BCA) value of ≤8 at 24 h was used as a primary cut-off indicating inhibition of bacterial growth. A new approach was used to determine synergy, indifference and antagonism based on the number of objects (bacteria) in the images. Static time–kill experiments were performed for comparison.

Results: The time–kill experiments showed synergy with 12 of 36 regimens, most frequently with colistin plus rifampin. BCA values ≤8 consistently correlated with 24-h bacterial concentrations ≤6 $\log_{10}$ CFU/mL. The classification of combination effects agreed with the time–kill results for 33 of 36 regimens. In three cases, the interactions could not be classified with the microscopy method because of low object counts.

Conclusions: Automated time-lapse microscopy can accurately determine the effects of antibiotic combinations. The novel method is highly efficient compared with time–kill experiments, more informative than checkerboards and can be useful to accelerate the screening for combinations active against multidrug-resistant Gram-negative bacteria. **W. Ungphakorn, Clin Microbiol Infect 2017;▪:1**

Introduction

Infections caused by carbapenemase-producing *Enterobacteriaceae* (CPE) are increasingly encountered [1–3] and are associated with high mortality [4,5]. CPE commonly harbour acquired resistance to multiple antibiotic classes leaving few effective therapeutic options [3]. Antibiotic combinations are often used for these infections supported by the results of observational studies [6,7]. Yet, clinical evidence is scarce and *in vitro* studies are needed to provide information on which antibiotics should be combined to optimize the antibacterial activity. Enhanced bacterial killing against CPE with combinations of two or three antibiotics has been shown using the checkerboard or time–kill methods [6,8–10]. Importantly, synergy has been observed also against bacteria that are highly resistant to the individual drugs used in the combination and cannot be predicted based on single-drug exposures. Therefore, a large number of regimens must be tested to discover potentially useful combinations.

The oCelloScope (Philips BioCell A/S, Allerød, Denmark) is a novel method based on digital automated time-lapse microscopy in which images are taken repeatedly during experiments and processed using growth kinetics algorithms. The method has been evaluated for its ability to measure bacterial growth and can be

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https://doi.org/10.1016/j.cmi.2017.10.029
1198-743X/

used to test up to 96 antibiotic regimens simultaneously with an automated readout [11,12]. In a recent study the technique showed consistent results with the time–kill method in the evaluation of single antibiotics against Gram-negative bacteria [11].

In this study, we assessed the feasibility of time-lapse microscopy to determine antibiotic combination effects against CPE. Four *Klebsiella pneumoniae* and two *Escherichia coli* were exposed to colistin, meropenem, rifampin and tigecycline, alone and in combination. Bacterial growth dynamics and drug interactions were determined with the oCelloScope and static time–kill experiments to allow a comparison of results between methods. We discuss the advantages and limitations of the novel method in relation to the standard methods for synergy testing and its potential to accelerate the screening for antibiotic combinations active against multidrug-resistant pathogens.

Materials and methods

Bacteria and media

Six clinical CPE isolates provided by the Public Health Agency of Sweden were used: four *K. pneumoniae:* ARU603 (VIM), ARU614 (OXA-48), ARU616 (KPC) and ARU639 (NDM) and two *E. coli*: ARU635 (NDM) and ARU636 (OXA-48). Cation-adjusted Mueller–Hinton (MH-II) (BD Diagnostics, Sparks, MD, USA) broth and agar plates were used for all experiments. In the oCelloScope experiments MH-II broth was filtered through a 0.2-μm filter (Filtropur S 0.2; Sarstedt AG & Co., Nümbrecht, Germany) before use to remove particles that may affect the analysis. Broth for the tigecycline experiments was freshly prepared on the day of use.

Antibiotics

Antibiotics were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Stock solutions were prepared by dissolving colistin and meropenem in sterile distilled water and dissolving rifampin and tigecycline in DMSO to a concentration of 10 000 mg/L. The following clinically relevant antibiotic concentrations were used in the time-lapse microscopy experiments: colistin, 0.125, 0.25, 0.5, 1, 2 and 4 mg/L [13]; meropenem, 1, 8, 16 and 32 mg/L [14]; rifampin, 1, 4, 8 and 16 mg/L [15]; and tigecycline, 0.5, 1, 2 and 4 mg/L [16]. In the time–kill experiments colistin at 0.5 mg/L was tested in combinations with meropenem at 8 and 32 mg/L, rifampin at 4 and 16 mg/L and tigecycline at 1 and 4 mg/L.

MIC determination and β-lactamase genes

Values of MICs were determined in triplicate by the gradient test method (Etest, bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions and interpreted using EUCAST definitions [17]. Whole genome sequencing was performed using Illumina paired-ends reads HiSeq sequencing (Illumina Inc., San Diego, CA, USA) and fastq files uploaded to ResFinder [18] to detect β-lactamase genes.

Time-lapse microscopy experiments

A single bacterial colony was inoculated and incubated overnight in MH-II broth at 37°C with 190 rpm shaking. The culture was diluted 100-fold, incubated for 1.5 h to achieve exponential growth phase and diluted 20-fold to obtain starting inocula of approximately 10^6 CFU/mL. Samples for viable counts were taken before each experiment to ensure that adequate bacterial concentrations were achieved. Antibiotics were added to a clear flat-bottomed 96-well microplate (Greiner Bio-One GmbH, Frickenhausen, Germany) and inoculated with the start culture to a total volume of 200 μL/well. Microplates were covered with highly transparent quantitative PCR film (Sarstedt AG & Co., Nümbrecht, Germany) and placed in the oCelloScope. The oCelloScope system was placed inside a Thermo Scientific Heratherm Incubator (Thermo Fisher Scientific Inc., Göteborg, Sweden) set to 37°C.

The bottom search method was used to determine the best focus for each well. During the experiments, ten images for each well were taken every 15 min for 24 h with an image distance of 4.9 μm, yielding a scan length of 405 μm and a scanned volume of 0.1 μL. Images were analysed with the background corrected absorption (BCA) algorithm [11,12]. The BCA algorithm detects bacterial growth by calculating a threshold pixel value to separate objects from background in the image and the bacterial growth curve is based on changes in the number of object pixels. The illumination time was set to 0.2 ms and the illumination level to 150 but was adjusted to 180–220 (depending on the concentration) for wells containing rifampin to compensate for the darker colour of this antibiotic in order to obtain similar initial BCA values. The 24-h images were visually checked for false increases in BCA due to evaporation or condensation, resulting in darker images without bacterial growth. When detected, data were discarded and the experiments repeated. All experiments were performed in five replicates.

Analysis of time-lapse microscopy data

A BCA value of ≤8 at 24 h was used as a primary cut-off value indicating inhibition of bacterial growth [11]. For experiments with the same antibiotic concentrations as in the time–kill experiments the 24-h images were further analysed by counting the number of objects (bacteria) in the scan area of each well using a segmentation process. Raw images were processed by correction of the background to reduce an uneven illumination. Marr-Hildreth [19] and Gauss filters (kernel 5 × 5) were applied to smooth the images. To find the points of maximal grey-level gradient a second derivative filter (Laplace filter, kernel 5 × 5) was added. Object pixels were separated from the background pixels by global thresholding [20],

Table 1
β-lactamase genes, MIC values and antibiotic susceptibility classifications according to EUCAST clinical breakpoints

Strain	β-lactamase genes[a]	MIC (mg/L) and susceptibility classification			
		CST	MEM	RIF[b]	TGC
Klebsiella pneumoniae ARU603	bla_{SHV-1}, bla_{CMY-4}, $bla_{CTX-M-15}$, **bla_{VIM-19}**	0.125 (S)	>32 (R)	>32	1.5 (I)
K. pneumoniae ARU614	bla_{TEM-1B}, $bla_{CTX-M-15}$, **bla_{OXA-48}**	0.125 (S)	16 (R)	>32	0.25 (S)
K. pneumoniae ARU616	$bla_{SHV-129}$, **bla_{KPC-2}**	32 (R)	>32 (R)	>32	0.19 (S)
K. pneumoniae ARU639	bla_{TEM-1A}, $bla_{CTX-M-15}$, bla_{CMY-4}, **bla_{NDM-1}**	0.125 (S)	>32 (R)	>32	0.38 (S)
Escherichia coli ARU635	bla_{TEM-1B}, $bla_{CTX-M-15}$, **bla_{NDM-1}**	0.125 (S)	32 (R)	32	0.094 (S)
E. coli ARU636	bla_{TEM-1B}, **bla_{OXA-48}**	0.19 (S)	0.25 (S)	24	0.19 (S)

Abbreviations: MIC, minimum inhibitory concentration; CST, colistin; MEM, meropenem; RIF, rifampin; TGC, tigecycline.
[a] Carbapenemase genes marked in bold.
[b] No clinical breakpoints exist for rifampin against *Enterobacteriaceae*.

Please cite this article in press as: Ungphakorn W, et al., Automated time-lapse microscopy a novel method for screening of antibiotic combination effects against multidrug-resistant Gram-negative bacteria, Clinical Microbiology and Infection (2017), https://doi.org/10.1016/j.cmi.2017.10.029