

DOUTORAMENTO

CIÊNCIAS MÉDICAS

Colistin susceptibility testing and therapeutic drug monitoring – applications of flow cytometry technology

Daniela Fonseca e Silva

D

2025

**Colistin susceptibility testing and therapeutic drug monitoring –
applications of flow cytometry technology**

Daniela Santos Fonseca e Silva Pinto de Freitas

Instituto Ciências Biomédicas Abel Salazar

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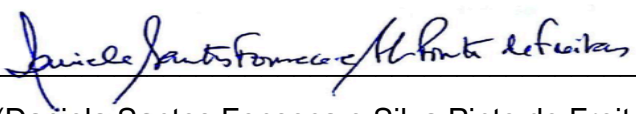
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Dedico este trabalho aos meus pais e aos meus dois filhos.

DECLARAÇÃO DE HONRA

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras de atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto plágio constitui um ilícito académico.



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LIST OF ABBREVIATIONS

ARGs	Antibiotic resistance genes
AST	Antimicrobial susceptibility
ATCC	American Type Culture Collection
ATU	Area of Technical Uncertainty
BC	Blood cultures
BM	Molecular Biology
BMD	Broth microdilution
C-FDA	Carboxyfluorescein diacetate
CA	Categorical agreement
CBA	Chocolate Blood Agar
CE-IVD	Certification under the In-Vitro Diagnostic Devices
CHUP	Centro Hospitalar e Universitário do Porto
CI	Confidence interval
CINTESIS	Centro de Investigação em Tecnologias e Serviços de Saúde
CLED	Cystine Lactose Electrolyte Deficient Agar
CLSI	Clinical Laboratory Standards Institute
CMS	Colistin methanesulfonate sodium
CS	Colistin sulfate
CVMP	Committee for Medicinal Products for Veterinary Use
DEFI	Departamento de Ensino, Formação e Investigação
DHR	123-Dihidrorodamin
DIBAC	Diethylbarbituric acid
EA	Essential agreement
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicines Agency
ESBL	Extended-spectrum beta-lactamase
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FACS	Fluorescence activated cell sorting
FCT	Fundação para a Ciência e a Tecnologia
FP	False Positives
GNB	Gram negative bacteria
HCRI	Health care related infections
HPLC	High performance liquid chromatography
IACS	Infecções associadas aos cuidados de saúde
ICBAS-UP	Instituto de Ciências Biomédicas Abel Salazar Universidade do Porto
ICU	Intensive care unit
IM	Inner membrane

IP	Propidium iodide
ISO	International Organization for Standardization
k	Kappa
KPC	Klebsiella pneumoniae carbapenemase
L-Ara4N	4-amino-4-deoxy-L-arabinose
LPS	Lipopolysaccharide
LPSs	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight
MDR	Multidrug-resistant
ME	Major error
mE	Minor error
MIC	Minimum inhibitory concentration
NCTC	National Collection of Type Cultures
OM	Outer membrane
OXA-48	Oxacillinase-48
PA	Percentage of agreement
PD	Pharmacodynamics
PEtn	Phosphoethanolamine
PK	Pharmacokinetics
PS	<i>Pseudomonas aeruginosa</i>
R	Resistant
S	Susceptible
SM	Microbiology Service
SPSS	Statistical Product and Service Solutions
TDM	Therapeutic drug monitoring
TTR	Time to results
U	Urine
UK-NEQAS	United Kingdom Nacional External Quality Assessment Schemes
USA	United States of America
UTI	Urinary tract infection
VAP	Pneumonia associated with mechanical ventilation
VME	Very major error
VTEC	Verotoxigenic <i>E. coli</i>
WHO	World Health Organization
ZnO	Zinc oxide

ABSTRACT

The emergence of multidrug-resistant (MDR) Gram-negative bacteria that cause health care related infections (HCRI) is a growing problem worldwide. Colistin is an old drug that is used as a “last-line” therapy to treat infections caused by multidrug-resistant Gram-negative bacteria, when essentially no other options are available.

Classical microbiology susceptibility tests are based on the study of the growing ability of microorganisms in the presence of the drug, taking a long time to give results (24-48h). Additionally, regarding peculiar characteristics of this drugs disk diffusion methods, *Etest* and most automated tests pose technical problems difficult to solve.

Flow cytometry has been used with great advantages in Microbiology especially regarding antimicrobial susceptibility evaluation, allowing also the understanding of mechanism of action and/or resistance.

This thesis describes a new and fast flow cytometric protocol which seems to be an excellent tool for evaluation of colistin susceptibility performed on colonies directly from blood culture and urine samples, with high agreement with broth microdilution reference method, providing a minimum inhibitory concentration (MIC).

First of all, we made a study design/ optimization of the cytometric protocol to determine colistin susceptibility. Then we made a proof-of-concept comparing with reference methods and study the reproducibility of the method using the recommended statistical analysis. The performance validation was first done with pure colonies, then directly from positive blood cultures (BC) for Gram-negative bacteria as well as positive urine samples in order to avoid subculture steps; qualitative and quantitative results obtained using this new assay were compared to microdilution, the reference method.

This methodology is easy to perform in clinical labs and provides results in useful clinical time, being an alternative protocol.

RESUMO

O aparecimento de bactérias Gram-negativas multirresistentes (MDR) em infecções associadas aos cuidados de saúde (IACS) é um problema crescente em todo o mundo. A colistina é um medicamento antigo utilizado atualmente como terapêutica de última linha para tratar infecções causadas por bactérias Gram-negativas multirresistentes, quando não existem disponíveis outras opções terapêuticas.

Os testes clássicos de suscetibilidade aos antimicrobianos, baseiam-se no estudo da capacidade de crescimento dos microrganismos quando expostos ao antibiótico, demoram por isso, muito tempo a dar os resultados (24-48h). Para além disso, os métodos de suscetibilidade aos antibióticos por disco, Etest e até mesmo os métodos automáticos apresentam problemas técnicos de difícil resolução. A citometria de fluxo tem sido utilizada como uma mais valia na área da Microbiologia, principalmente no que diz respeito à avaliação da suscetibilidade aos antimicrobianos, permitindo perceber o seu efeito e até o mecanismo de ação e/ou resistência.

Esta tese descreve um novo e rápido protocolo de citometria de fluxo, que pode ser uma excelente metodologia para a avaliação da suscetibilidade à colistina em colónias puras, hemoculturas ou amostras de urina, fornecendo um resultado qualitativo e quantitativo (concentração inibitória mínima (CIM)) com elevada concordância com o método de referência (microdiluição em caldo),

O protocolo de citometria de fluxo para determinação da susceptibilidade à colistina começou pelo desenho do estudo e optimização do método. Em seguida foi realizada uma prova de conceito comparando o protocolo de citometria de fluxo com método de referência e determinada a reprodutibilidade. Realizou-se a análise estatística dos dados seguindo protocolos recomendados. Após validação da metodologia com colónias puras, prosseguiu-se o estudo para a análise de bactérias extraídas diretamente de hemoculturas (HC) positivas para bacilos de Gram-negativo assim como amostras de urina positivas evitando a sub-cultura; o resultado qualitativo e quantitativo foi comparado com a microdiluição (método de referência).

Esta metodologia é fácil de ser realizada em laboratórios clínicos e fornece resultados em tempo útil para o tratamento do doente, apresentando-se como uma solução alternativa para a determinação da susceptibilidade à colistina.

STRUCTURE

This thesis consists of eight chapters:

Chapter 1 – Introduction – Review of colistin's mechanism of action and resistance, as well as current and future challenges

Chapter 2 – Aims

Chapter 3 – Optimization of the flow cytometry protocol for evaluation of colistin susceptibility and validation of the determination of the minimum inhibitory concentration (MIC) by ultra-rapid flow cytometry assay in Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

In **Chapter 4** – Evaluation of colistin susceptibility using the flow cytometry assay directly from positive blood cultures (BC) in order to provide a faster response in patients with sepsis.

In **Chapter 5** – Evaluation of colistin susceptibility using flow cytometry assay directly on positive urines for Gram-negative bacteria using the automated system UF-1000i (Syxmex®).

Chapter 6 – Discussion of new methodologies present on the market along with advantages and disadvantages comparing them with flow cytometry. Addresses the future perspectives towards other polymyxin like polymixin B and the possibility of quantification the amount of colistin present to the blood of patients under colistin treatment.

Chapter 7 – Conclusion about this work, the methodology and how it may represent a disruptive alternative to reference method, which is difficult to perform in clinical labs.

INTRODUCTION

Colistin Update on Its Mechanism of Action and Resistance, Present and Future Challenges

Review article

The content of this chapter is published in *Microorganisms*, 2020,8(11),1716.

Daniela Fonseca e Silva was a main contributor, participating in literature review, manuscript writing and final editing.

ABSTRACT

Colistin has been extensively used since the middle of the last century in animals, particularly in swine, for the control of enteric infections. Colistin is presently considered the last line of defense against human infections caused by multidrug-resistant Gram-negative organisms such as carbapenemase-producer *Enterobacteriales*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Transferable bacterial resistance like *mcr*-genes was reported in isolates from both humans and animals. Researchers actively seek strategies to reduce colistin resistance. The definition of guidelines for colistin therapy in veterinary and human medicine is thus crucial. The ban of colistin use in swine as a growth promoter and for prophylactic purposes, and the implementation of sustainable measures in farm animals for the prevention of infections, would help to avoid resistance and should be encouraged. Colistin resistance in the human-animal-environment interface stresses the relevance of the One Health approach to achieve its effective control. Such measures should be addressed in a cooperative way, with efforts from multiple disciplines and with consensus among doctors, veterinary surgeons, and environment professionals. A revision of the mechanism of colistin action, resistance, animal and human use, as well as colistin susceptibility evaluation is debated here.

KEYWORDS

colistin; polymyxin; antimicrobial resistance; multidrug-resistant gram-negative organisms

1. Introduction

Colistin (Polymyxin E) is a polycationic peptide antimicrobial, discovered in 1949 in Japan, produced by *Bacillus polymyxa*. It belongs to the polymyxin class of antibiotics, with hydrophilic and lipophilic properties. The polymyxin group includes five different chemical compounds (polymyxins A, B, C, D, and E) [1,2]; only two polymyxins are used clinically: polymyxin B and colistin, also called polymyxin E [3]. For clinical use, two forms of colistin are available: a prodrug, colistin methanesulfonate sodium (CMS), for parenteral use, and colistin sulfate (CS) for oral, inhalator, or topical use [4].

It was first used in human and veterinary medicine in 1952, but between the 1970s and 1980s, its medical use was almost abolished, thereby persisting the veterinary use. In

recent years, colistin has begun to be used in humans as a last resource agent in the case of infections by multidrug-resistant Gram-negative bacilli, notably by carbapenemase-producing *Enterobacterales*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* [5–8]. The World Health Organization (WHO) and other government agencies such as Health Canada have reclassified colistin in the category of “very high importance for Human Medicine” [9,10].

Colistin is considered by the European Medicines Agency (EMA) as antibiotics critically important in human medicine - Category B - “Restrict”. This means its administration in veterinary medicine should be limited to reduce the danger to public health. Quinolones (fluoroquinolones and other quinolones), cephalosporins of either third and fourth generation (excluding those with beta-lactamase inhibitors), and polymyxins are drugs included in this category. Antibiotics in Category B should be reserved for treatment when antimicrobials in Categories C or D are not, from a clinical point of view, effective and there is no other alternative. In general, antibiotic utilization should rely upon the performance of an antimicrobial susceptibility test (AST), particularly those included in Category B [11].

2. Mechanism of Action

Colistin L-diaminobutyric acid, positively charged, binds through electrostatic interaction to the negatively charged phosphate groups of lipid A, an important component of lipopolysaccharide (LPS) of Gram-negative bacilli [12]. The lipid A plays a crucial role in bacterial permeability and exchange with cell exterior [6]. Colistin displaces the divalent cations of calcium (Ca^{2+}) and magnesium (Mg^{2+}) in a competitively way, impairing the LPS three-dimensional structure. Colistin then inserts its hydrophobic terminal acyl fat chain, causing an expansion of the external outer membrane (OM) monolayer. A permeabilization of OM occurs, allowing colistin to get through OM as a self-promotion. The synergy exhibited by colistin and other antimicrobials with hydrophilic properties such as β -lactamics, gentamicin, rifampicin, meropenem, and tigecycline is explained by this process [13]. The phospholipid bilayer of the inner membrane (IM), present only in Gram-negatives, loses its stability, because of the action of colistin by including hydrophilic groups in the fatty acid chains, changing its integrity and leading to its destruction, failing to maintain cellular content, leading to cell lysis [6]. Binding to lipid A, colistin also exerts an anti-endotoxin activity [1], preventing the induction of shock by endotoxin. Colistin essentially solubilizes the bacterial cell membrane, resulting in a bactericidal effect.

3. Colistin Resistance

Resistance to colistin can be explained by a variety of mechanisms. Until 2015, it was thought to be acquired solely via chromosomal point mutations. As LPS is the target of colistin, any change in it can change the behavior of colistin [7]. *Salmonella* and *E. coli* are able to modify LPS by changing lipid A through the biosynthesis of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtn). Its biosynthesis is associated with chromosomal mediated resistance, dependent on two-component response regulators and sensor kinase systems: PmrA/PmrB and PhoP/PhoQ [14–16]. The first system PmrA/PmrB also controls the pmr HIJKLM operon, which promotes the synthesis of N4-aminoarabinosis, which in turn, when bonding chemically the fractions of lipid A, changes the negative charge of the cell membrane by neutralizing the negatively charged phospholipids. This resistance mechanism is exhibited by *Pseudomonas aeruginosa* [17]. The resistance to colistin expressed by *Acinetobacter baumannii* is based on the suspension of LPS production. This absence of LPS production may result from the inactivation of a lipid A biosynthesis gene, lpxA, lpxC, or lpxD, and leads to resistance to colistin due to the absence of lipid A [18].

Regarding the resistance expressed by *K. pneumoniae* to polymyxins, its resistance mechanism is based on the inactivation of the mgrB gene and, in deregulation, the two-component regulatory systems PhoPQ and PmrAB. Inactivations in the mgrB gene were responsible for the exhibition of resistance by *K. pneumonia* to colistin. When the PhoP/PhoQ signaling system is activated, a small transmembrane regulatory protein, composed of 47 amino acids, is produced: the MgrB. This protein prevents the phosphorylation of PhoP, probably by suppressing the PhoQ kinase or promoting the phosphatase. As PhoP phosphorylation is found to increase the transcription of the mgrB gene and, on the other hand, MgrB inhibits this phosphorylation, then this protein is clearly a mediator of negative feedback in the PhoQ/PhoP signaling circuit [19,20].

Initially reported in China in 2015 [21], and later in Asia, Africa, Europe, and America [22–25], a plasmid mediated colistin resistance, *mcr-1* gene, was described in *Escherichia coli*. The *mcr-1* is an enzyme that changes lipid A present in LPS with a metabolite of phosphoethanolamine, suppressing its binding to colistin.

The chances of transferring bacterial resistance to colistin along with resistance to broad-spectrum cephalosporin are significantly raised by the fact that a single plasmid can co-harbor *mcr-1* together with the extended-spectrum beta-lactamase gene (ESBL). This poses huge challenges when treating infections with Gram-negative etiology. A *mcr-1*

gene located chromosomally was detected in two colistin-resistant *E. coli* isolates, collected from calves [26].

Later, in Belgium, a new colistin resistance gene, *mcr-2*, was also discovered, carried by a plasmid in *E. coli* isolates from porcine and bovine biologic samples and also co-harboring ESBL genes [27].

Interestingly, the coexistence of several *mcr* genes on *E. coli* does not necessarily mean a significant difference in terms of minimum inhibitory concentration (MIC), compared with resistant isolates of *Salmonella* only carrying the single plasmid *mcr-1* gene [28]. The *mcr-1* gene found in resistant *Enterobacterale* isolated from swine was often associated to a low level of resistance; MICs of 4 or 8 mg/L were reported for most isolates, which are only 2-4 times higher than the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint (2 mg/L) [21,28,29] or Clinical Laboratory Standard Institute (CLSI). Strains with minimal inhibitory concentrations inferior to 2 mg/L are considered susceptible by EUCAST protocol and intermediate by CLSI; susceptible category was recently eliminated by CLSI.

Colistin resistant bacteria also share resistance to other types of antibiotics used such as aminoglycosids; tetracycline; sulfonamide and trimethoprim; lincosamide; β -lactamics; quinolones; and third generation cephalosporins involving different mechanisms of resistance such as enzymatic, efflux, impermeability, or point mutations [29–33].

Meanwhile, seven additional *mcr* homologues (*mcr-3* to *mcr-9*) have been identified in *Enterobacterales* [34,35] and PCR tests have been developed to enable their detection [36]. This mechanism of resistance can be acquired during therapy and easily transmitted, increasing the spread of resistance.

The emergence of resistance to colistin, one of the few remaining therapeutical alternatives for patients infected with *K. pneumoniae* resistant to carbapenems and other important antimicrobial groups, is thus a major concern, notably among humans.

4. Epidemiology of Colistin-Resistance

According to the European Centre for Disease Prevention and Control (ECDC), in 2017, isolates resistant to colistin represented 8.5% (2.4% of all reported *K. pneumoniae* isolates and only sporadically on *E coli*). Greece and Italy were the countries responsible for the vast majority (88.5%) of these reports [37].

On the other hand, the same data source reported in 2016 that only 51.3% of all *P. aeruginosa* isolates were colistin susceptible. Concerning *Acinetobacter* spp., colistin susceptibility data reach up to 51.3% of all isolates [37].

However, the ECDC in 2018 repeated the warnings that these findings may not be representative for Europe as a whole and should be interpreted with caution because of the low number of isolates tested, the relatively high proportion of isolates from areas of high resistance, and the technical complexities involving colistin susceptibility tests [38]. The possibility of transmission of colistin resistant *E. coli* between species is possible, particularly from swine [39] or from pets [40] with close relationships with humans.

5. Colistin Susceptibility Testing Assays

For both individual and epidemiological purposes, it is essential to have accurate antimicrobial susceptibility testing (AST) regarding bacteria isolated from infected humans and from animals. Because colistin binds to several laboratory materials, many technical issues are raised and the results of colistin susceptibility can be tricky and often incorrect. A survey in 2017, among laboratories providing data, revealed that the majority of the laboratories did not test colistin susceptibility locally or used methods that are not recommended [38].

The Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), American and European organizations respectively, responsible for the standardization of AST laboratory protocols, joined efforts to produce colistin susceptibility rules. They have issued recommendations confirming that microdilution is, for the time being, the only susceptibility testing method valid for colistin.

Several difficulties related to the methodology arise in the assessment of susceptibility to colistin. For performing an AST, the 20776-1 standard method of microdilution (BMD) is the reference method and the only one now validated for *Enterobacterales*, *P. aeruginosa*, and *Acinetobacter* spp. Culture media, colistin formulation, and even the kind of plastic used on microplates impact and were standardized. However, such a method is cumbersome and requires a long time to achieve a result (minimum of 24-48 h). Susceptibility testing by other methods, including agar dilution, disk diffusion, gradient diffusion, and automated methods (such as Vitek2, Phoenix), is not recommended, making most of the available epidemiological data not accurate. Newer methods including molecular approaches still need optimization; while they can detect the few,

known resistance genes, they cannot formally be considered a susceptibility assay. The detection of resistance genes, such as *mcr*, means that the strain is resistant to colistin, although its absence does not necessarily mean susceptibility. A novel method based upon flow cytometry that allows AST determination in a maximum of 2 h versus 2 days, directly from positive blood cultures or from colonies (2 h versus 1 day in that case), can change the diagnosis paradigm [41,42]. Quick AST reports by the microbiological lab, in an era of an increase of antimicrobial resistance, are thus urgently needed [43].

6. Colistin: Human Use

In recent years, with the scarcity of antimicrobials options available, there is a growing interest in the deprecated antibiotic, the colistin. Colistin has established itself as a last resort therapy in infections of gram-negative multidrug-resistant (MDR) etiology. Because of the nephrotoxicity and neurotoxicity exhibited, colistin was abandoned in the early 1980s, but its resurgence as a resource therapy in critically ill patients is taking place. Regarding its intravenous use, we can identify three milestones in the course of colistin: 1950–1970, for gram-negative infection in general; 1990–2000, in cystic fibrosis with gram-negative MDR infections; and after the passing of the millennium until present time, against infection of gram-negative MDR etiology.

International consensus recommendations for colistin therapy provide a guide for its optimal clinical use [44]. It is often the last line of defense against multidrug-resistant Gram-negative bacteria, namely, *Enterobacterales*-carbapenemase producers [45,46], *Pseudomonas* spp., and *Acinetobacter* spp. Despite being Gram-negative, pathogens such as *Brucella* sp., *Burkholderia cepacia*, *Helicobacter pylori*, *Edwardsiella* sp., *Moraxella catarrhalis*, *Neisseria* sp., *Proteus*, *Providencia*, *Serratia*, and *Stenotrophomonas mallei* do not generally show susceptibility to colistin and, with regard to *Campylobacter* sp., its susceptibility has a wide range of variation. However, it is particularly used in critical clinical conditions such as bacteremia or sepsis and pneumonia associated with mechanical ventilation (VAP) in the intensive care unit (ICU). For other several clinical conditions, such as for urinary tract infections, meningitis, osteomyelitis and joint infections, infections of the gastrointestinal tract, pneumonia, abscess, pyoderma and/or soft tissues infections, and eye and ear infections, colistin is seen as an alternative treatment. On the other hand, myasthenia gravis and hypersensitivity to polymyxin are conditions in which the use of colistin is contraindicated and there are no scientific data to support or establish safety in its use in pregnant and lactating women. Colistin should be administered carefully with dose correction accordingly and tight surveillance in patients with renal impairment because of its

nephrotoxicity. Intravenous polymyxins have been evaluated for the treatment of serious multidrug-resistant *P. aeruginosa*, *Acinetobacter baumannii*, and *Enterobacterales* infections [1].

Intravesical administration of colistin in therapies of persistent urinary tract infections with *Acinetobacter baumannii* etiology [47] has been described, as well as intrathecal administration in the case of meningitis because of the low permeability to colistin by the hemato-encephalic barrier [48].

Selective decontamination of the digestive tract (SDD) is a practice used in intensive care, in which colistin can be used orally along with a non-prolonged treatment of broad-spectrum parenteral antimicrobials. Despite the existence of scientific data supporting this intensive care practice to reduce bloodstream infections and mortality, when it extends over time, the rising of resistance to colistin among ESBL-producing *K. pneumoniae* isolates has been seen [49–51]. Recent studies with contradictory conclusions show that this practice is far from the consensus [52,53].

Some authors propose inhaled colistin as monotherapy or associated with systemic therapy in the treatment of pneumonic processes or in chronic pulmonary infection of multi-resistant Gram-negative etiology [54].

Pharmacodynamics (PD) and pharmacokinetics (PK) bring emerging data on colistin as monotherapy. It is very difficult to entrust that monotherapy will produce enough plasma levels, with the real possibility and danger of promoting the resistance. For this reason, colistin combination therapy seems to increase clinical success and reduce resistance emergence. In vitro experimental data also support the preference for a combination therapy, particularly updated studies through the usage of dynamic models. Several studies, both in vitro and in vivo (mouse model), have pointed out that the association of colistin with other antibiotics, such as rifampicin and imipenem, has a better performance compared with colistin alone in the case of multidrug-resistant Gram-negative bacteria [55,56]. However, Lagerbäck et al. recommended that such an approach should be further studied in vivo, considering that colistin combination therapy should be reserved for critical ill patients with multidrug-resistant Gram-negative bacteria pneumonia [56]. This therapeutic approach still needs additional clinical studies, addressing its real effectiveness and, not less important, its resistance potential impact.

Lee et al. conducted a study reporting that the combined therapies with colistin and carbapenem, tigecycline, or rifampicin were more effectively in comparison with the

colistin monotherapy treatment regarding positive carbapenemase *Klebsiella pneumoniae* [57]. The difficulty lies in defining clinical protocols supported by pharmacodynamics and toxicodynamics for combined colistin therapies that allow the highest possible bacterial lethality with the lowest chance of toxicity, and simultaneously prevent the emergence of resistance.

The new cephalosporins associated with beta-lactamases inhibitors like ceftazidime/avibactam could be an alternative for some carbapenemase producing bacteria (especially *Enterobacterales* KPC and OXA-48), and clinicians are left in a challenging situation: either they keep treating with old well-known drugs or they adopt new antibiotics with insufficient evidence [58].

Regarding *Pseudomonas*, a synergetic activity of colistin with ceftazidime has been reported as well as the combination of colistin with rifampicin and amikacin. The combination of colistin with rifampicin has also provided a synergetic antibactericidal effect for infections caused by *Pseudomonas aeruginosa* MDR [1].

Both in vitro and in vivo studies are clear to corroborate that antimicrobial resistance expands when bacterial agents are exposed to inferiorly unsuitable colistin gradients, observed in a significant proportion of patients [59], particularly in isolated heteroresistant strains such as, for example, those of *Acinetobacter baumannii* [60].

The association with carbapenem, doripenem, or meropenem, as well as with several other antimicrobials, such as rifampicin, glycopeptides, daptomycin, and fusidic acid, generally ineffective in Gram-negatives, revealed a synergistic effect against *Acinetobacter baumannii*. The effective mechanism of such unlikely combined therapies rests on the polymyxin effect, changing the outer membrane permeability, allowing or/and making easier, despite being large molecules, the entry of antimicrobials into the cytoplasm [46].

Discrepancies now can be found on the antimicrobial susceptibility report; recently, CLSI (2000) abolished the classification of susceptible for all gram-negative bacteria *Enterobacterales*, *Pseudomonas*, and *Acinetobacter*, only naming as intermediate or resistant, although EUCAST (2000) still maintains the susceptible category.

Therapeutic drug monitoring of colistin is warranted because its pharmacokinetics is quite variable and because its therapeutic window is narrow [61]. This aspect makes the management of the colistin treatment challenging, stressing the need for the availability

of practical and simple methods with a short response time, including a drug monitoring assay.

7. Colistin: Veterinary Use and Its Impact

At present, colistin is an antibiotic still widely used in veterinary medicine, mostly in pigs, for the oral treatment of intestinal infections caused by *Enterobacterales* [62]. The administration of antibiotics like colistin in animals supported the growing of modern farm animal productions, allowing successful weaning, higher animal density, and probably higher viability of economic control of pathologies caused by *E. coli* infections such as that caused by verotoxigenic *E. coli* (VTEC) [22]. Colistin is poorly absorbed through the gastrointestinal tract. This fact stresses the emergence of colistin resistance as a result of selective pressure upon the intestinal microbiota [22]. Pigs treated with colistin had overall higher proportions of resistant isolates compared with pigs not treated [62]. Colistin is also used orally in calves for the treatment of gastrointestinal diseases caused by Gram-negative bacteria. This use may also explain the isolation of colistin-resistant bacteria from calves, despite the lack of solid data about the treatment of these animals with colistin [32].

Notably, the most common use of colistin in swine industrial production worldwide is the oral route and for prophylactic purposes [63]. Colistin was mainly administered via feed, but also through drinking water [64]. This practice involves medicating all animals in the same farm, resulting in treating animals with clinical symptoms together with healthy ones. The European Medicines Agency (EMA) made a categorization for colistin use, which was reviewed in 2016 [64]. Colistin therapies with prophylactic purposes carry a high risk in the emergence of resistance, and thus should be banned. Its clinical use should be limited to enteric infections caused by susceptible non-invasive *E. coli* and supported by an AST whenever possible [43,64].

Although colistin, in oral treatment, is generally used as monotherapy [22], there are some pharmaceutical forms on the market with colistin, under the sulfate salt chemical form, that allow combined therapy. The most common association with other antimicrobials is with β -lactamics and mainly amoxicillin [65]. In vitro data show the combination of amoxicillin with colistin reveals a synergistic or additive effect against pathogenic *E. coli* of avian source [66]. Combinations of colistin and amoxicillin plus zinc oxide (ZnO) during the pre-weaning and weaning periods incorporated in the swine feed were also used [67]. A study carried out in fecal samples of weaned piglets showed that the combination of three antimicrobials, colistin, bacitracin-zinc, and chlortetracycline,

inhibits the rise in tet resistance genes (tetX, tetC, tetL, and tetW) [68]. Despite the suppression in tet resistance genes, no data exist regarding resistance to colistin after using this combined therapy. Because of a lack of evidence, the combined therapy cannot be scientifically sustained, particularly among pigs. Considering all the available updated knowledge, the Committee for Medicinal Products for Veterinary Use (CVMP), basing the decision on the precautionary principle, recommended the Market Authorization withdrawal for all Market Authorization Holders and all veterinary pharmaceutical formulas associating colistin and other antibiotics [64].

The use of colistin as a growth promoter of farm animals, still adopted specially in Asia, should be banned. Low sub-inhibitory concentrations of antibiotics used to improve animal growth have been linked to the emergence of antibiotic resistance [22]. Notably, antimicrobials for animal growth promotion can generally be purchased without veterinary control even in the European Union. High sanitation, by controlling the microbial burden in the farm, is a key factor to help to ban antimicrobial misuse.

As a general attitude with all antimicrobials, but in particular with colistin, the veterinary surgeon should ensure that the antimicrobial prescribed is applied strictly for the treatment of sick animals as recommended in compliance with label instructions. Any deviations from the EMA guidelines should be justified and recorded [11,64]. In this context, extra-label use of colistin such as, for example, in countries where this antibiotic is not approved in swine, must take place within a valid veterinary justification relying upon AST reports and without the availability of other therapeutic alternatives.

Further research is mandatory in order to determine the withdrawal period allowing the risk reduction of animals sent to slaughter harboring colistin resistant bacteria carrying *mcr* genes.

Animal-to-human transmission of *mcr-1* resistance raises issues concerning the consequences of using colistin in veterinary medicine (in pet treatment, farm animal's production, and its penetration in the consumption food chain) in terms of human health [39]. *mcr-1* might be present in the environment and transmissible by several routes to humans. Therefore, it could also add to the potential transferring of the *mcr-1* gene from animal to human. Such transfer routes require additional and more comprehensive studies.

The veterinary surgeon should seek that colistin use only targets clinical disease and consider banning its use whenever feasible. Educational and awareness campaigns for

pig farmers and employees are essential. Records detailing colistin use in animals are equally critical as they provide support to allow the development of policies and guidelines and to assess the effect of possible interventions. It has been shown that colistin-resistant *E. coli* was isolated from healthy individuals without prior medical colistin use [69].

Additionally, reports from Italy, the second country using colistin adjusted for biomass under exposure in European Union [64], describe the detection of colistin-resistant *E. coli* from wild rabbits (*Oryctolagus cuniculus*) and wild hares (*Lepus europaeus*) that were not previously treated with colistin [70]. Consequently, wildlife may also play a role in colistin resistance, being a potential reservoir in the environment, and contribute to its transmission to other animals and/or humans through contaminated food and water or by direct human and animal contact.

Apart from the previous isolation of bacteria resistant to colistin from manure, water, migratory birds, and vegetables [23,71–73], the ecotoxicity of this antimicrobial and its impact is an issue to take into consideration [74,75]. The colistin, at high levels, present in swine farms' wastewater, is related to the toxicity exhibited in the bacteria responsible for oxidizing ammonia [74]. The biotransformation of xenobiotic substances along with the chemical transformation of ammonia into nitrites in wastewater treatment plants is ensured by these bacteria responsible for the oxidation of ammonia [74]. The significant damage inflicted on the intestinal epithelium and the greater expression of stress-related genes in the earthworm *Eisenia fetida* are other rising ecotoxic markers revealing the environmental toxicity caused by the elimination of colistin in wastewater [75].

It should be remembered that administered antibiotics are not 100% metabolized by animals or humans; many are excreted in an active form via the feces or urine. This fact is even more marked with oral administration of colistin, which is very poorly absorbed by the gastrointestinal tract [22].

Composting methods have been reported to be able to eliminate on average 50–70% of some antimicrobials, such as chlortetracycline, monensin, and tylosin [76], also helping to reduce the relative amounts of the *bla*_{TEM}, *sul3*, and *erm*(B) genes in manure [77]. Unfortunately, they do not work in reducing the amounts of colistin or *mcr* genes in pig manure. The method in which manure is handled or treated has a variable impact on the effectiveness of reducing antibiotic resistance genes (ARGs) in pig manure; that is, aerobic biofiltration of manure seems to be more effective to reduce *erm*(X) than other ARGs such as *erm*(F), *erm*(B), and *tet*(G), while mesophilic anaerobic digestion and

lagoon storage reduced none of these ARGs [78]. A great deal of controversy still involves the effectiveness of these biological treatments of manure, such as lagoons and composting, in ARG reduction [76]. To ensure the destruction of resistant bacteria and ARGs present in animal and human waste is mandatory in further research. It is necessary to ensure the effectiveness of waste treatment processes, particularly with regard to pig manure. The agricultural role of swine manure in soil fertilization and the absence of regulations and guidelines should be considered to control and monitor the use of swine manure [22].

8. Conclusions

An interdisciplinary collaboration and communication between all the specialists involved is necessary to develop a universal strategy that fosters the reduction of the emergence of resistance to colistin in human, veterinary medicine, and the environment. The scientific community, experts, and government authorities urge for a reduction of colistin usage, recommending its prescription only for the treatment of infections as a last resource under strict circumstances. Colistin use should involve a scientific evaluation of the effectiveness of other drugs and implement alternative strategies against infections caused by Enterobacterales whenever possible, avoiding antimicrobials at all, particularly in preventive strategies. The *mcr-1* gene was isolated on four continents and, most worrying, from sources other than food animals, such as the environment as well as from human isolates. In addition, some *E. coli* isolates harboring the plasmid-encoded *mcr* genes, now known up to *mcr-9*, were also ESBL or carbapenemase enzyme producers. All such facts cause great concern about the eventual loss of colistin effectiveness regarding the treatment of multidrug-resistant Gram-negative bacilli in humans. Epidemiological data could not be accurate owing to technical issues involved in the susceptibility evaluation. Rapid and accurate ASTs are urgently needed. This highlights the need for the judicious use of colistin in order to avoid the development of pan-resistant strains that are not bound by land borders and do not require a passport or visa.

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Aims

The specific aims of this research project were:

1. Optimization of a flow cytometric protocol for detection the susceptibility to colistin by flow cytometry and validating it in pure colonies of Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter* spp.
2. Determination the susceptibility to colistin using the flow cytometry protocol directly from positive blood cultures from Gram-negative bacilli avoiding sub-culture.
3. Determination the susceptibility to colistin using the flow cytometry protocol directly from urine samples positive for Gram-negative bacilli avoiding sub-culture.

3.1 - Optimization of a novel flow cytometric protocol for the study of colistin activity

The subject of this chapter was partially presented in an oral session in 28th ECCMID; April 21-24, 2018; Madrid, Spain: “**Optimization of a novel flow cytometric protocol for the study of colistin activity**”; Daniela Fonseca e Silva, Rita Teixeira-Santos, Ana Dias, Sara Oliveira, Inês Oliveira, Maria Helena Ramos, Acácio Gonçalves Rodrigues, Cidália Pina-Vaz;

1. Abstract

Introduction

Colistin (CS), also known as polymyxin E, has been increasingly used as a “last-line” option to treat infections caused by multidrug-resistant (MDR) Gram-negative bacteria. Due to the alarming global rise of antimicrobial resistance, colistin has regained clinical relevance despite its well-known toxicity. However, conventional antimicrobial susceptibility testing (AST) methods for colistin are characterized by long turnaround times, typically ranging from 24 to 48 hours. Moreover, several challenges have been reported in performing reliable *in vitro* susceptibility testing for colistin, owing to factors such as drug adsorption to plastic surfaces and suboptimal diffusion in standard assays. Given these limitations, there is a critical need for rapid, accurate, and reliable testing methods to guide therapy in infections caused by MDR pathogens. The present study aims the optimization of a novel flow cytometric protocol to evaluate qualitative and quantitative cell lesions induced by CS in clinical useful time. The goal is to provide clinically actionable results in a much shorter time frame, achieving a short time-to-result (TTR).

2. Material and methods

This study involved the analysis of three bacterial strains: two colistin-susceptible strains (*P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922) and one colistin-resistant strain [*E. coli* NCTC 13846 (*mcr-1*-positive)]. Bacteria were incubated during 15, 30 and 60 min, at 37°C with colistin (C4461, Sigma) at concentrations ranging between 0.25 to 8 µg/mL, and stained with different fluorescent dyes designed to probe different physiological parameters, such as membrane lesion, membrane potential, metabolic activity and the generation of reactive oxygen species (ROS). Cells were then analyzed in a BD Accuri™ C6 Plus Flow Cytometer (Fig 1), enabling high-throughput, multiparametric analysis of individual bacterial cells.

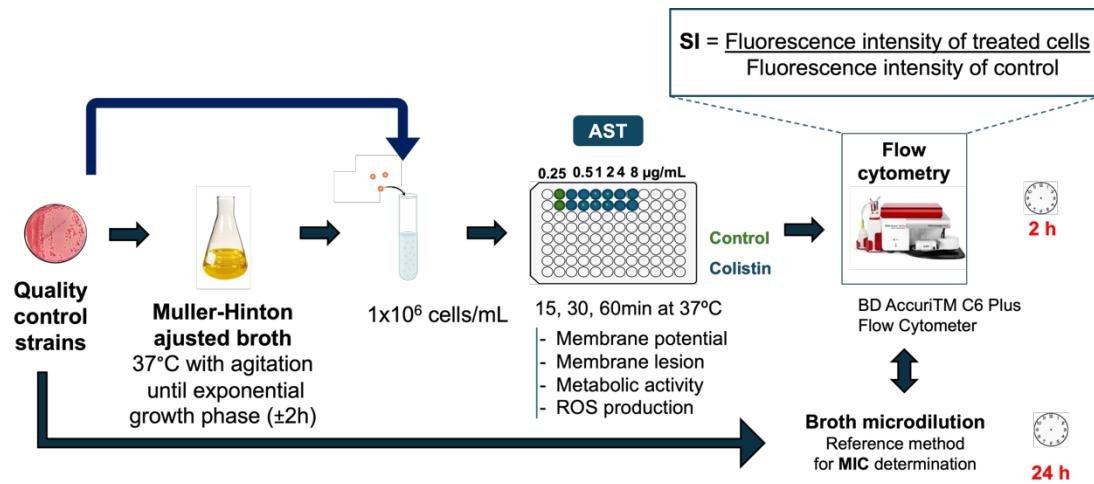


Figure 1. Schematic representation of the workflow.

For each experimental conditions, the fluorescence intensity of treated cells was compared to that of untreated control cells (control). (Fig 2 - 4).

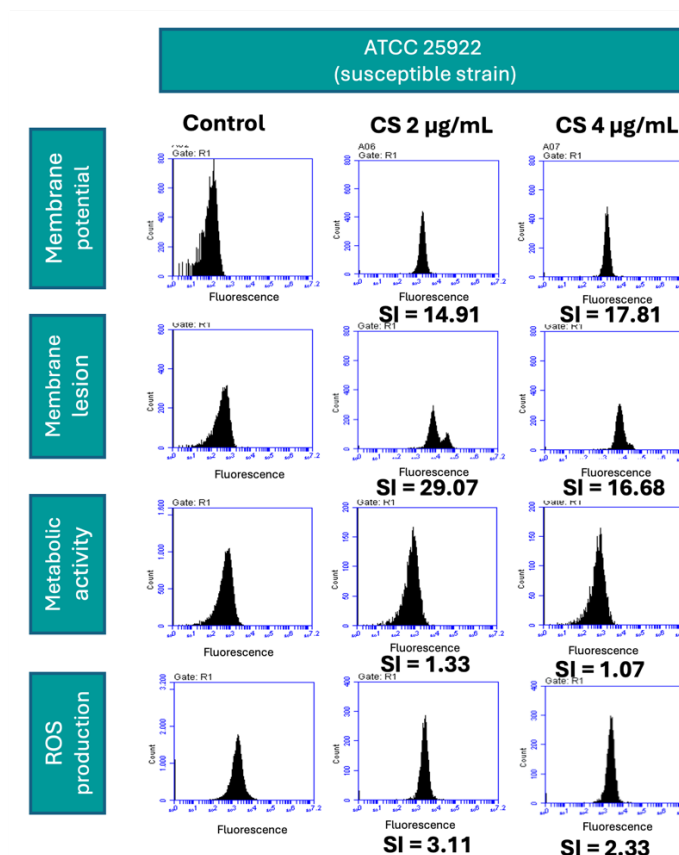


Figure 2. Fluorescence distribution for a susceptible strain (ATCC 25922) after incubation with 2 and 4 µg/ml colistin during 60 minutes, and staining with different dyes. CS: colistin; SI: staining index.

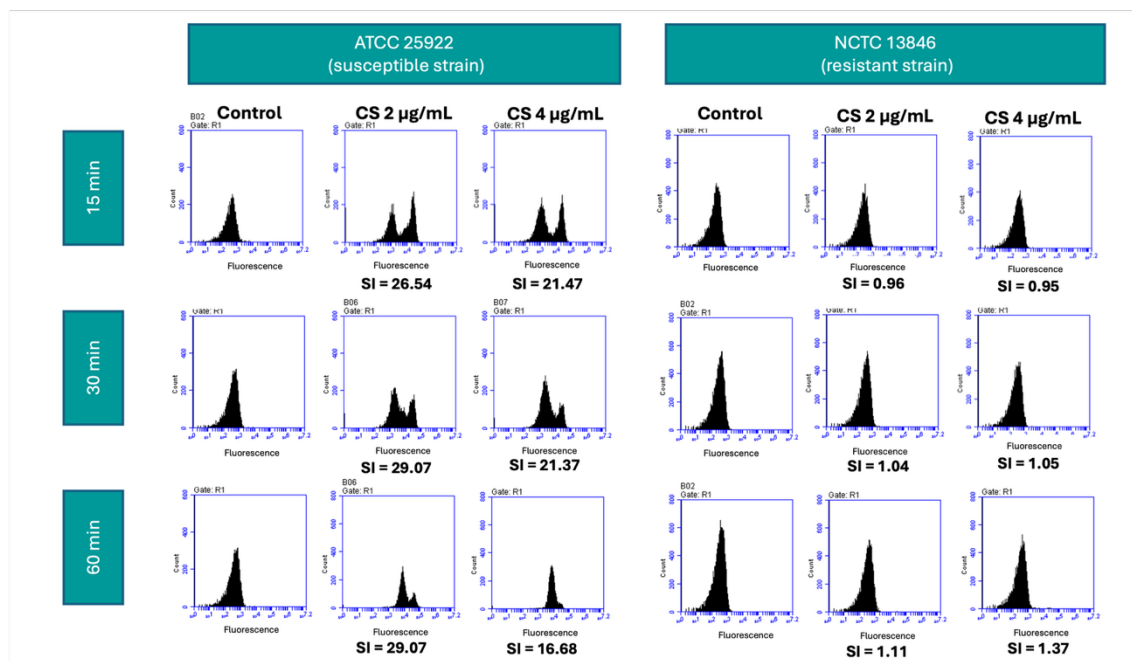


Figure 3. Fluorescence distribution for one susceptible strain (ATCC 25922) and one resistant strain (NCTC 13846) after incubation with 2 and 4 µg/ml colistin during 15, 30 and 60 min, and staining with a membrane lesion dye. CS: colistin; SI: staining index.

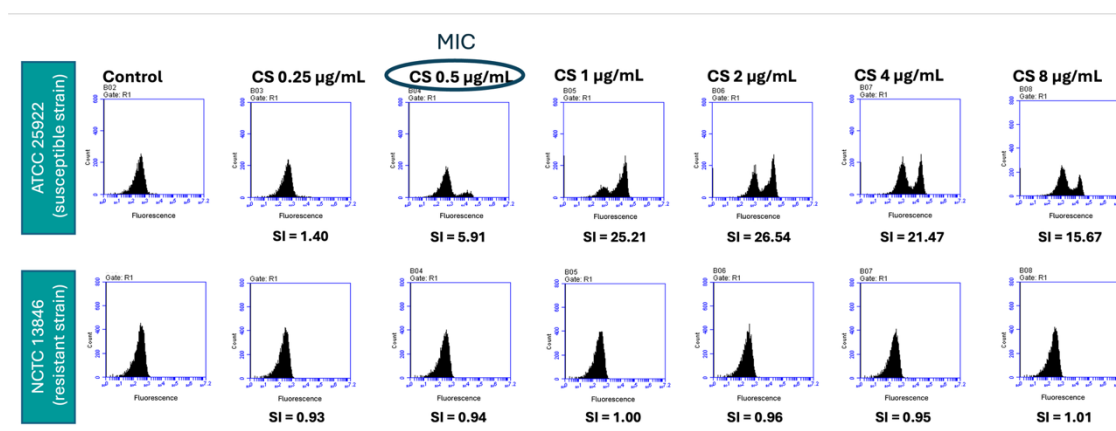


Figure 4. Fluorescence distribution for one susceptible strain (ATCC 25922) and one resistant strain (NCTC 13846) after incubation with colistin during 15 min and staining with a membrane lesion dye. CS: colistin; SI: staining index.

Statistical analyses were performed to evaluate differences between incubation times using the Wilcoxon test. Comparison of cytometric profiles between susceptible and resistant strains was carried out using the Kruskal–Wallis test. A p -value <0.05 was considered statistically significant.

3. Results

Exposure of the colistin-susceptible strains to colistin resulted in notable alterations in cellular physiology, as evidenced by flow cytometric analysis. These alterations included increased membrane permeability, membrane depolarization, impaired metabolic activity, and elevated ROS production. In contrast, the resistant strain (NCTC 13846) exhibited minimal changes in these parameters, even after 60 minutes of incubation with colistin.

Importantly, significant differences were observed between susceptible and resistant strains as early as 15 minutes of incubation, particularly when assessing membrane lesion markers ($p < 0.001$). and this effect revealed to be dose-dependent. Moreover, cytometric data showed a high agreement (1.00) with the minimum inhibitory concentration (MIC) values of colistin previously determined according to CLSI and EUCAST protocols (EN ISO 20776-1, 2016), specially using a membrane lesion stain.

4. Conclusion

In conclusion, this study proposes a novel and rapid method for colistin susceptibility testing based on flow cytometry, capable of providing accurate MIC determination within a clinically meaningful time frame. By enabling susceptibility profiling in less than 2 hours, this protocol holds potential to significantly improve clinical decision-making and facilitate earlier initiation of targeted antimicrobial therapy in patients with multidrug-resistant Gram-negative bacterial infections.

3.2 - Ultra-rapid flow cytometry assay for colistin MIC determination in Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

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This manuscript is presented in a thesis for the first time.

Daniela Fonseca e Silva was a main contributor, participating in methodology, data analysis, manuscript writing and final editing.

ABSTRACT

Objectives: Both EUCAST and CLSI recommend broth microdilution for antimicrobial susceptibility testing of colistin, but this method is cumbersome and takes 16-24 h to give results. Our objective was to evaluate a rapid quantitative colistin MIC susceptibility assay based on flow cytometry analysis (FAST-colistin MIC) in comparison with standard broth microdilution assay.

Methods: One hundred and sixteen Gram-negative bacilli (78 *Enterobacterales*, 28 *Pseudomonas aeruginosa* and 10 *Acinetobacter baumannii*) were studied in parallel using standard broth microdilution following EUCAST recommendations and FASTcolistin MIC kit. In the last one, a bacteria suspension (0.5 MacFarland) was prepared, diluted in Muller-Hinton broth, incubated in the susceptibility panel containing different colistin concentrations (range 0.125-64 mg/L) with a fluorescent probe and incubated 1 h at 35°C. After that, a flow cytometry analysis using CytoFLEX (Beckman) was performed. Using a dedicated software (BioFAST) an automated MIC result was obtained after 1.5 h. Performance evaluation was performed according to the ISO standard 20776-2. Reproducibility and repeatability, categorical (CA) and essential agreement (EA), and lot-to-lot variation and operator-to-operator variability, as well as time to results were determined.

Results: Overall, 100% CA (CI 97-100%) and 95.7% EA (CI 90-98%) was obtained with high repeatability (100%; CI 80-100%) and reproducibility (97%; (CI 83-99%)). Absence of lot-to-lot variations or differences in the operators' performance was observed.

Conclusions: FASTcolistin MIC is an accurate, reliable and ultra-rapid method (1 h incubation versus 24 h) for susceptibility testing of colistin of common Gram-negative bacilli recovered in clinical laboratories.

1. Introduction

The emergence of multidrug-resistant Gram-negative bacteria worldwide has led to a re-evaluation of previously discontinued antibiotics, such as colistin, a polymyxin, introduced in early 1950s [1-3]. It is the 'last-line' therapy to treat infections caused by multidrug-resistant Gram-negative bacteria when no other antibiotic options are available [4]. It binds to lipopolysaccharides and phospholipids of the outer cell membrane. Unfortunately, its utility is limited by the nephrotoxicity [5], even at potentially therapeutic

concentrations, and an overall lack of understanding on how to optimize its administration [6-8]. Antimicrobial susceptibility testing (AST) is crucial in the selection of therapy but colistin presents several technical issues. Adhesion to pre-treated plastic laboratory consumables can significantly influence the result and most of commercial tests, including disc diffusion method, gradient tests and automated systems are no longer recommended [9]. Broth microdilution is the only validated colistin AST method recommended by EUCAST and CLSI [10]. ISO-standard reference testing required the use of cation-adjusted Mueller-Hinton Broth, polymyxin sulfate salts and untreated polystyrene trays with no additives. Clinical laboratories rarely perform microdilution, and some friendly tests have recently become available with acceptable performance [9]. Despite this situation, these simplified assays are preformed manually, are growth based requiring at least 18- 24 hours. Flow cytometry assays developed by FASTinov, a spin-off of Porto University, describe a new approach already patented (USA, US9290790B2; Europe, EP2714922B1; and Japan, JP6587643B2) [11]. We describe the evaluation of the FASTcolistin MIC, a rapid AST assay based on flow cytometry for MIC determination of colistin, in 1.5 hours from isolated colonies.

2. Material and methods

Strains

One hundred-sixteen Gram-negative bacilli: 78 Enterobacterales (12 *Escherichia coli*, 38 *Klebsiella pneumoniae*, 21 *Enterobacter spp*, 3 *Proteus spp*, 2 *Morganella morganii*, 1 *Providentia rettgeri* and 1 *Serratia marcescens*), 28 *Pseudomonas aeruginosa* and 10 *Acinetobacter baumannii* isolates belonging to the cryobanked (-80°C) collection of Microbiology Department of Porto Medical School (Portugal) were studied. Most of them are clinical samples and 16 belong to American Type Culture Collection (ATCC) (*E. coli*: 25922, 8739, 35218, 13846, 13476; *K. pneumoniae*: BAA 1705, BAA 1706, 700603, 13443; *S. marcescens*: 14756, *Enterobacter cloacae*: CCUG 59627, 13048; *P. rettgeri*: BAA 2525; *P. aeruginosa*: 27853, BAA 2108; *A. baumannii*: BAA 2108).

Colistin susceptibility testing

Minimal inhibitory concentration (MIC) was performed according 2 different methods: i) Microdilution reference method. All strains were inoculated in standard polystyrene

plastic MIC strips with serial concentration of sulphate salt of colistin (range 0.125-64 mg/L); MICs were calculated by visual inspection after 24 hours of incubation at 35°C (EUCAST and CLSI protocols) [10] and ii) the FASTcolistin MIC kit. It includes a susceptibility testing panel, a dehydrated 96 multi-well microplate with colistin at serial concentrations (0.125-64 µg/ml) and a fluorescent dye, a dedicated software (BioFAST SW) and Instructions for use. Briefly, a bacterial suspension is prepared from 8-24 hours pure colonies at 0,5 McFarland and diluted (1/50) in filtered Muller Hinton broth cation adjusted. The AST panel is inoculated by adding 100 µl of the bacterial suspension to each of the 12 wells. As quality control, a negative well with non-treated cells, and a positive control with citric acid, to assure fluorescent dyes performance was included. After 1-hour incubation at 35°C with shaking and protected from light the panel is analysed through flow cytometer analysis, the CytoFLEX (Beckman Coulter). Cell fluorescence intensity, as well as morphological changes by light scatter changes are recorded in an FSC (flow cytometer standard) file. BioFAST SW analyses the FCS files provided by the flow cytometer and reports the MIC, as well as the phenotype according to CLSI or EUCAST criteria (susceptible, intermediate or resistant). The number of events, the light scatter values and the fluorescence intensity of each well are incorporated on the algorithm and gave the results based on previously determined cut-off values. Cut-off values for FC were calculated previously using ROC curves [12] and introduced on the software.

Performance evaluation of the FASTcolistin MIC kit

Repeatability and reproducibility were determined by using triplicate testing with ten strains with varying colistin MIC values on-scale, from independent inoculums, according ISO 20776-2. Variability between three different assay lots and three different operators were compared using 3 ATCC strains with different MIC values (ATCC13846 - MIC 4 µg/ml, ATCC27853 - MIC 1 µg/ml and CCUG59627-MIC 0,5 µg/ml). Categorical agreement (CA) and essential agreement (EA) between both methods was performed.

Statistical analysis

The percentage agreement (PA) was calculated in order to evaluate repeatability and reproducibility, compare lot-to-lot and different operators.

3. Results

Most *E. coli* and *K. pneumoniae* tested with colistin MIC equal or higher to 4 mg/L, were positive for *mcr-1*; 6 *Kl. pneumoniae* R to colistin were negative for all *mcr* genes, 1 *E. coli* was positive for *mcr-2* and another for *mcr-4*. Two *Pseudomonas* strains were S to colistin but presented efflux. Colistin reference MICs determined by BMD for the 116 strains are highly correlated with the FASTcolistin MIC test results (Fig. 1). CA was 100% (97-100%) and EA was 95.7% (CI 90-98%). Thirty-five strains gave MIC values higher than BMD but only 4 with more than 1 dilution above, and 20 below with only 1 with more than 1 dilution apart (Fig. 1).

		FASTcolistin MIC (mg/L)										
Reference method MIC (mg/L)		<=0,125	0,25	0,5	1	2	4	8	16	32	64	>64
	<=0,125	16	1	4								
	0,25	1	3	11								
	0,5		2	11	12							
	1			11	9	3						
	2				1	2						
	4						2	2				
	8							4	1			
	16								2			
	32									1	1	
	64								1	1	4	
	>64										3	7

Figure 1. Correlation between MIC determined by FASTcolistin MIC (flow cytometry antimicrobial susceptibility assay) and reference method (BMD) of 116 Gram-negative bacilli. MICs within essential agreement (within +/- 1 dilution of reference MIC) are highlighted in grey and MIC identical in both tests are within boxes. EUCAST and CLSI breakpoints are shown as lines. MIC ≤ 2 mg/l means Susceptible for EUCAST and Intermediate for CLSI; MIC ≥ 4 is resistant for both EUCAST and CLSI protocols.

Flow cytometry results obtained from different phenotypes are represented on Fig. 2. An increase on the intensity of the fluorescent, meaning an increase on the membrane permeability indicates the antibiotic toxicity to the bacterial cells. The minimal concentration of colistin that increases the intensity of fluorescence above the previously optimized cut-off value, is the reported MIC. R strains did not show any increase of fluorescence intensity, at least until the breakpoint concentration independently of the underlined mechanism of resistance. Strains with efflux were correctly classified. Reproducibility has percentage agreement of 96,6% (PA = 0.966) (CI 83-99%) and the

repeatability was excellent, 100% (PA = 1) (CI 80-100%). No difference from operators or between different lots was observed, (PA = 1).

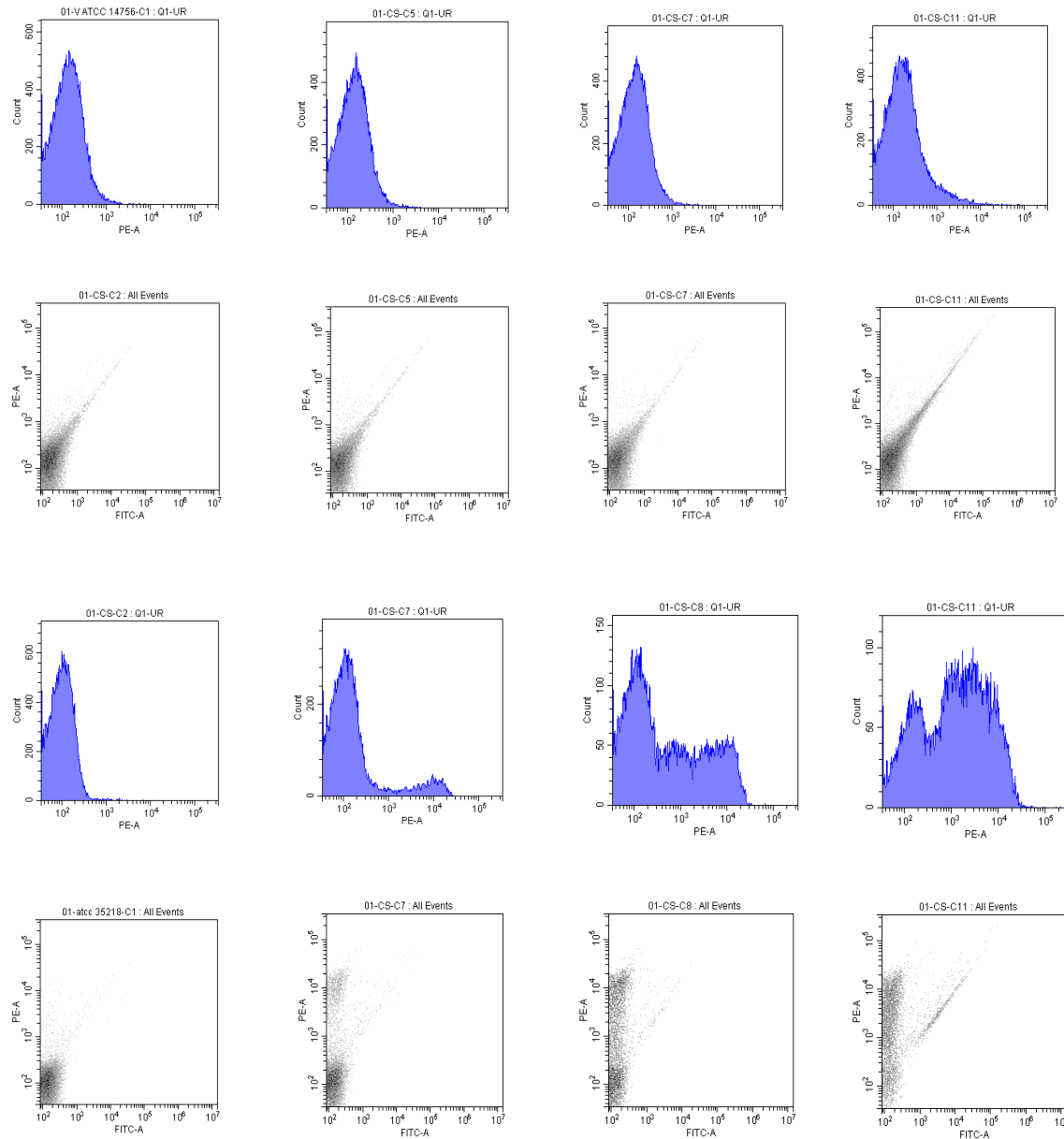


Fig. 2. Flow cytometry graphics of a colistin-resistant strain (A. *E. coli* ATCC 14756) and a susceptible-strain (B. *E. coli* ATCC 35218), both exposed to colistin. Fluorescence displayed by stained cells are represented in columns 1. Bacterial cells non-exposed to colistin; 2. cells exposed to 1 mg/L of colistin; 3. cells exposed to 2 mg/L of colistin (breakpoint concentration); 4. cells exposed to 32 mg/L of colistin. A drift of the population to the right on the histogram (blue) and to the top in case of the dot plot is evident on the susceptible strain.

4. Discussion

Over the past decades, growth in antimicrobial resistance has overtaken the development and market of new antimicrobial [13] drugs. Also, rapid AST is urgently need and could facilitate rational and target therapy, avoiding empiric approaches, often using broad spectrum or multiple drug therapeutic strategies leading to increase the multidrug triggers. Innovative and rapid AST systems have been described, which can be divided in genomic, proteomic and phenotypic methods [13]. We previously described a qualitative AST based on flow cytometry for main antibiotics including colistin directly from positive blood cultures [14] with excellent results [11]. The assay described here is quantitative and qualitative, simple to perform, accurate and provide a rapid AST report directly from colonies. Due the mechanism of action of colistin, cells can be used directly from colonies, avoiding the need of exponential growth phase, as with beta-lactam antibiotics [15]. The CA and EA results are even higher than several MIC commercial tests previously evaluated by Matuschek et al. [9]. For this new assay a flow cytometry is mandatory, a tool under explored but with a great potential in Microbiology.

In conclusion, we present a reliable and simple tool for ultra-rapid susceptibility testing of colistin based on flow cytometry.

Author contributions

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication. Cidália Pina-Vaz and Daniela Fonseca e Silva designed the study, analysed the results and wrote the paper. Ana Silva Dias, Rosário Gomes, Inês Oliveira and Ferdinando Andrade collaborate on the experiments; Maria Helena Ramos, Acácio G Rodrigues and Rosário Gomes collaborated of the discussion of the results and on the writing of the manuscript.

Transparency declaration

Rosário Gomes, Ana S Dias, Sara-Cruz and Ines M-Oliveira are employees of FASTinov, S.A. Cidália Pina-Vaz and Acácio G Rodrigues, are co-founders of FASTinov, S.A. However, the study was designed and partially executed in the Microbiology Department of Faculty of Medicine/CINTESIS of Porto University. Other authors do not declare conflict of interest.

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Evaluation of rapid colistin susceptibility directly from positive blood cultures using flow cytometry assay

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This manuscript is presented in a thesis for the first time.

Daniela Fonseca e Silva was a main contributor, participating in methodology, data analysis, manuscript writing and final editing.

ABSTRACT

Accurate assessment of colistin susceptibility is crucial with the increasing number of multidrug-resistant Gram-negative bacteria and simultaneously increasing colistin resistance. Both EUCAST and CLSI recommend broth microdilution (BMD) to determine colistin susceptibility, however it is cumbersome and growth-dependent. In this study, a rapid flow cytometry method (FASTinov®) to determine colistin susceptibility directly from positive blood cultures (BCs) was evaluated. BCs were spiked with 204 Gram-negative bacilli (137 Enterobacterales, 35 *Pseudomonas* spp. and 32 *Acinetobacter baumannii*) at a concentration of 2×10^3 cells/bottle, inoculated with human donor blood and incubated until flagged positive. As quality control strains, two susceptible (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two resistant (colistin-resistant *mcr*-1-positive *E. coli* NCTC 13846 and *Serratia marcescens* ATCC 14756) were used. Bacteria were extracted according to assay instructions and were incubated for 1 h at 37 °C with 2 and 4 mg/L colistin and a fluorescent dye, previously optimised. Cells were analysed on CytoFLEX (Beckman Coulter) and Accuri™ C6 Plus (BD Biosciences) flow cytometers. Colistin susceptibility results were automatically provided by BioFAST software (FASTinov®) and compared with those obtained with standard BMD. Overall categorical agreement between this new flow cytometry method and BMD was 99.0%. No very major errors were detected as well as no discrepancies between both flow cytometers. Here we describe a rapid and accurate assay for colistin susceptibility directly from positive BCs with a turnaround time of 2 h versus 48 h required for BMD. This method represents an accurate alternative to standard BMD.

Keywords

Colistin, polymyxin, flow-cytometry, Enterobacteriaceae and *Pseudomonas*, MDR microorganisms

1. Introduction

Blood culture (BC) represents the gold standard for identification of microorganisms causing bloodstream infections or septicemia [1]. For antimicrobial susceptibility testing (AST) of the microorganisms involved, the current reference procedure requires positive BCs to be subcultured on solid media, thus usually requiring 48–72 h from BC positivity

to the availability of AST results to clinicians. Empirical broad-spectrum therapy is commonly initiated to circumvent the reporting delay, representing a costly approach that often fails to effectively target the correct microbe to achieve therapeutic success.

FASTinov[®] (Porto, Portugal) has developed a disruptive flow cytometry method to evaluate several phenotypic lesions induced by antimicrobial drugs over bacterial cell populations [2,3]. This novel assay allows the evaluation of AST in a turnaround time of 1–2 h. It can be used directly from colonies, saving almost 1 day, but also directly from positive BCs, thus saving ≥ 2 days in result reporting time [4,5]. This technology provides the potential for real-time antibiotic decision-making, thus ensuring more rapid initiation of targeted therapy.

Colistin is currently considered one of the few 'last-line' therapies for multidrug-resistant Gram-negative bacilli. It is a membrane-active drug with several human toxicity side effects [6]. The reference methodology for colistin AST is minimum inhibitory concentration (MIC) determination by standard broth microdilution (BMD) [7]. However, colistin BMD has several technical issues, thus limiting its use in most routine clinical laboratories. Recently, a joint Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing (CLSI-EUCAST) working group investigated colistin BMD and gave specific recommendations [8]. Nevertheless, this method remains cumbersome, time consuming and difficult to perform in routine daily practice, requiring ≥ 48 h from the moment that the BC is flagged positive. Consequently, different methods to determine colistin susceptibility have been proposed, including colorimetric and mass spectrometry assays [9–11]. Here we describe a rapid susceptibility test for colistin clinical categorization based on flow cytometry methodology that takes < 2 h to perform from the time the BC turns positive.

2. Methods

2.1. Blood cultures samples

BC bottles (Becton Dickinson, USA) were spiked with 204 Gram-negative bacilli, comprising: 137 Enterobacterales, including 12 intrinsically resistant isolates presenting colistin MICs > 32 mg/L (8 *Proteus* spp., 1 *Serratia marcescens*, 2 *Morganella morganii* and 1 *Providencia rettgeri*) and 11 isolates with acquired colistin resistance with colistin MICs in the range 4–8 mg/L (10 *Klebsiella pneumoniae* and 1 *mcr-1*-positive *Escherichia coli*); 35 *Pseudomonas* spp., including 5 resistant isolates with colistin MICs of 8 mg/L ($n = 4$) or 4 mg/L ($n = 1$); and 32 *Acinetobacter baumannii*, including 1 resistant to colistin

with an MIC of >64 mg/L. BCs were inoculated with non-treated human blood from donors and were incubated until obtaining a positive flag [12]. As quality control strains, two colistin-susceptible (*E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two colistin-resistant (*mcr-1*-positive *E. coli* NCTC 13846 with a colistin MIC of >32 mg/L and *S. marcescens* ATCC 14756 with a colistin MIC of >16 mg/L) were used.

2.2. Extraction of microorganisms and incubation with colistin and a fluorescent probe

A gel tube (BD Vacutainer® Barricor LH Plasma; Becton Dickinson) was filled (≈5 mL) with the positive BC and the bacteria were extracted after two centrifugation steps (1500 × *g* for 5 min and 1 min, respectively). The pellet was resuspended in brain–heart infusion broth (Liofilchem, Roseto degli Abruzzi, Italy) and a 0.5 MacFarland suspension was prepared and diluted 10-fold. The cells were then incubated for 1 h at 37 °C with 2 mg/L and 4 mg/L colistin according CLSI and EUCAST protocols for AST and with a fluorescent probe that only labels bacteria with cell membrane damage.

2.3. Flow cytometry analysis

Subsequently, cells were analysed in parallel both in a CytoFLEX (Beckman Coulter, USA) and an Accuri™ C6 Plus (BD Biosciences, USA) flow cytometer. Data analysis of the cytometric files was automatically performed by BioFAST software, a dedicated software developed by FASTinov®.

2.4. Colistin broth microdilution routine procedure and antimicrobial susceptibility testing flow cytometry evaluation

Spiked positive BCs were subcultured on blood agar plates for 24 h [7]. BMD AST was performed as recommended and the results were interpreted according both to EUCAST and CLSI guidelines. For the EUCAST protocol, the clinical breakpoints are susceptible ≤2 mg/L and resistant >2 mg/L for Enterobacterales, *Pseudomonas* spp. and *Acinetobacter* spp., and an Area of Technical Uncertainty (ATU) at 4 mg/L for *Pseudomonas* spp. [13]. For the CLSI protocol, breakpoints are susceptible ≤2 mg/L and resistant ≥4 mg/L for *Pseudomonas* spp. and *Acinetobacter* spp. No breakpoints are yet established for Enterobacterales and only epidemiological cut-off values suggested that

wild-type isolates have colistin MICs ≤ 2 mg/L and non-wild-type isolates have colistin MICs ≥ 4 mg/L [14].

2.5. Data analysis and comparison between methods

Categorical agreement was calculated according to ISO standard 20776-2:2016, as well as very major errors (VME) and major errors (ME). VME rates were calculated using the total number of resistant isolates as the denominator, whereas ME rates were calculated using the total number of susceptible isolates as the denominator [15]. Minor errors were not calculated as no intermediate category has been established. Results obtained from CytoFLEX and AccuriTM C6 Plus were compared using Cohen's kappa (κ).

3. Results

Fig. 1 illustrates the distribution of fluorescence obtained with three control strains as an example of two susceptible strains (one Enterobacterales and one *Pseudomonas*) and one colistin-resistant strain, treated or not with the two colistin concentrations (2 mg/L and 4 mg/L) and stained with a membrane potential fluorescence marker after 1 h of incubation. In the case of the colistin-resistant control strain (*mcr-1*-positive *E. coli* NCTC 13846), there were no differences between the fluorescence displayed by cells exposed to colistin and the internal control (stained bacteria unexposed to colistin) (Fig. 1B). Regarding colistin-susceptible strains, there was an increase in fluorescence intensity compared with the internal control (Fig. 1A,C). The increase in fluorescence indicates cell membrane damage (shift to the right). Thus, differences in fluorescence intensity allow the discrimination of resistant from susceptible strains.

The overall categorical agreement between this new rapid flow cytometric method and the standard BMD reference method was 100% for Enterobacterales and *A. baumannii* and 94.3% for *Pseudomonas* spp. with one ME (1/30) with a BMD MIC of 2 mg/L and one ME in a strain with a BMD MIC of 4 mg/L, an ATU according to EUCAST criteria but resistant for CLSI. Regarding the strain classified as ATU by the reference method, it was categorized as resistant by flow cytometry. Moreover, there were no differences between susceptibility results obtained from CytoFLEX and AccuriTM C6 Plus ($\kappa = 1.00$).

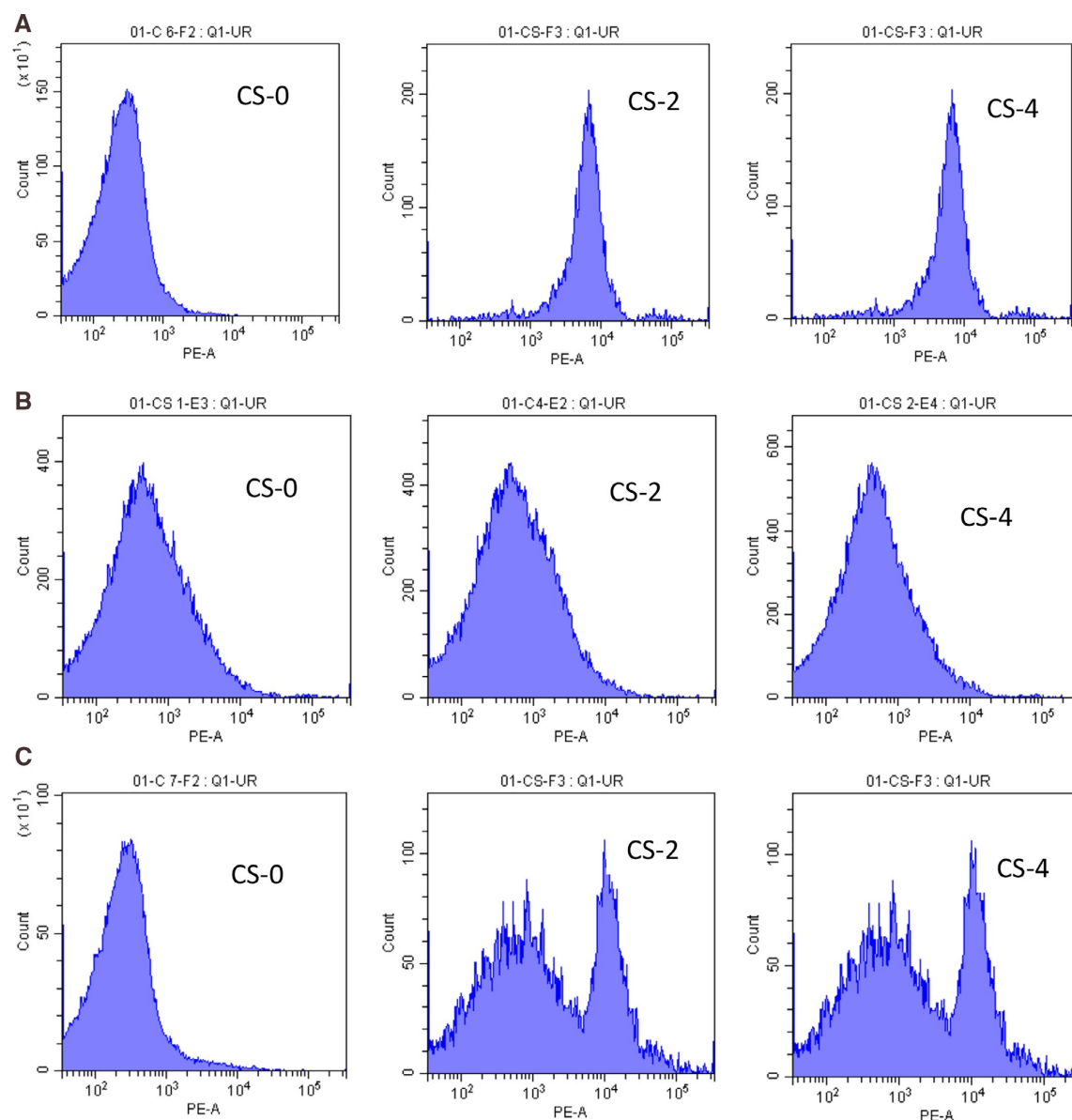


Fig. 1. Flow cytometry histograms of (A) *Escherichia coli* ATCC 35218 (colistin-susceptible), (B) *E. coli* NCTC 13846 (colistin-resistant strain *mcr-1*-positive) and (C) *Pseudomonas aeruginosa* ATCC 27853 (colistin-susceptible). Fluorescence displayed by control cells non-exposed to colistin (CS-0) and stained with fluorescent dye; cells exposed to 2 mg/L colistin and stained (CS-2); and cells exposed to 4 mg/L colistin and stained (CS-4)

4. Discussion

In this study, we validate the performance of a rapid flow cytometric assay for the study of colistin susceptibility directly from positive BCs. Indeed, it was possible to discriminate between clinically susceptible and resistant isolates by quantitative assessment of fluorescence intensity differences in bacterial cells exposed to different concentrations of colistin. Among colistin-susceptible isolates, there was an increase in fluorescence of treated cells compared with the control, whilst in colistin-resistant isolates there were no differences between treated bacteria and the control. The increase in fluorescence intensity signifies that the cell membrane was damaged as a result of colistin exposure according to the mechanism of action of the drug. Colistin acts on the cell membrane of Gram-negative bacteria, leading to disruption of the outer membrane with consequent leakage of intracellular contents and subsequent bacterial death [6]. Accordingly, susceptible bacteria treated with colistin exhibit a measurable higher fluorescence.

Different probes have been proposed to test AST using flow cytometry [16]. According to the colistin mechanisms of action, a probe that only penetrates when the cell membrane is permeable appears to be the most appropriate to study susceptibility to this particular drug. This approach allows much earlier detection of response compared with growth-dependent assays for AST. Notably, it should be stressed that the described flow cytometric test evaluates the antibiotic-dependent bacterial cell membrane lesion, thus being a functional assay, with the advantage of not being growth-dependent, contrary to other existing methodologies [17]. In addition, flow cytometric assay is very rapid and accurate method, requiring <2 h after the BC becomes positive, with excellent correlation with the current reference method for colistin AST.

In conclusion, we propose a novel rapid diagnostic assay for colistin susceptibility directly from positive BCs. We believe that its implementation in clinical microbiology laboratories should impact the patient management of bacteremia in this era of an increasing number of multidrug-resistant Gram-negative bacteria and simultaneously increasing colistin resistance. This methodology represents a valid alternative to the current cumbersome BMD reference method, which is difficult to perform in most routine clinical laboratories. It was developed to be easy to perform, needing no special abilities. The results are provided by the developed software without the need for great expertise in flow cytometry. Identification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) directly from BC coupled with the FASTinov® flow cytometry assay would help to effectively decrease reporting time in septic patients.

Declarations

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Competing Interests: None.

Ethical Approval: Not required.

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Rapid determination of colistin susceptibility by flow cytometry directly from positive urine samples

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This information is presented in a thesis for the first time.

Daniela Fonseca e Silva was a main contributor, participating in methodology, data analysis, manuscript writing and final editing

ABSTRACT

Objectives: Urinary tract infections caused by Gram-negative bacteria (GNB) are among the most common infections and a significant cause of sepsis. The increasing prevalence of multidrug-resistant (MDR) bacteria poses challenges to empirical treatment. Colistin may be used as a last-resort antibiotic for treating MDR infections, but this requires the rapid determination of susceptibility to colistin. Traditional susceptibility testing methods can take up to 48 h, and there are specific challenges in determining colistin susceptibility. This study evaluates a novel, rapid method for determining colistin susceptibility directly from positive urine samples using the FASTcolistin MIC kit from FASTinov®. A total of 100 urine samples positive for Gram-negative bacilli when screened by the UF-1000i system were included in this study. After a simple sample prep, the same bacterial suspension was used for identification on MALDI-TOF and inoculated in the FASTcolistin MIC panel for our AST; after incubation at 37 °C for 1 h, it was analyzed via flow cytometry using a CytoFLEX cytometer (Beckman Coulter, Brea, CA, USA). The categorical susceptibility to colistin according to EUCAST or CLSI standards as well as the MIC values were given by bioFAST software (bioFAST 2.0). The essential agreement (EA) and bias were calculated. Different species of Enterobacterales, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. were correctly identified by MALDI-TOF directly from the FASTcolistin MIC sample prep. The essential agreement between the two methods was 99%, with a bias of -17%. Both identification and susceptibility were obtained in less than 2 h. This study presents a rapid and accurate method for determining colistin MIC directly from urine samples. The shortness of time required to produce a result, 2 h versus 48 h with the conventional methods, will significantly impact treatment decisions, especially in urinary tract infections difficult to treat.

Key words

Colistin, Rapid antimicrobial susceptibility test, Flow cytometry, Antimicrobial resistance, Urinary tract infections

1. Introduction

Urinary tract infections (UTIs) are the second most common type of infection world- wide, affecting 150 million people each year, with significant morbidity and high med- ical costs (e.g., it has been estimated that the economic burden of recurrent UTIs in the United States is more than USD 5 billion each year) [1], and with a higher incidence in females. Gram-negative aerobic bacteria (GNB), particularly *Escherichia coli* (responsible for 75–90% of UTIs), are the predominant causative agent, followed by *Klebsiella pneumoniae*, *Proteus*, and *Pseudomonas* spp. [2].

Up to 50–70% of women experience a UTI within their lifetime, and of these, 40% will have a recurrence [3]. The use of urinary catheters and underlying conditions such as dia- betes further increase the risk of recurrence. UTIs are a common cause of sepsis, accounting for 19.8% of cases [3]. Antimicrobial resistance (AMR) in UTIs pathogens is a growing concern, exacerbating the challenge of treating both primary and recurrent infections. The prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) is increasing, threatening the efficacy of carbapenem antibiotics, commonly considered the last line of defense against multidrug-resistant strains (MDRs) of Gram-negative bacteria [4]. This rise in AMR is linked to the widespread and often inappropriate use of antibiotics in both medical and agricultural sectors [5]. The growing prevalence of AMR leads to longer treatment times, higher healthcare costs, and increased morbidity, especially in recurrent infections where treatment options become limited [6]. The need for alternative strategies is leading people to look back to old therapies like colistin.

Colistin (polymyxin E) is used as a last resort for the treatment of critically ill patients infected with multidrug-resistant (MDR) GNB infections [7]. These patients are usually ex- tremely ill and require a rapid AST result. Colistin can be used intravenously, inhaled, used intrathecally, or even given through intravesical irrigation [8]. Determining colistin suscep- tibility is considered a challenge in traditional methods as it adheres to plastic (automated methods, disks, and the E-test are not recommended by EUCAST or CLSI guidelines), with broth microdilution being the only gold standard for determining the colistin MIC (minimum inhibitory concentration), although laborious and time consuming [9].

Rapid antimicrobial susceptibility tests (ASTs) for colistin using FASTinov technology from isolated colonies [10] and directly from positive blood cultures [11] presented excellent results and saved 1–2 days, respectively. FASTinov technology is a disruptive technology, which uses flow cytometry as a basis, not dependent on bacterial growth, in

contrast to most ASTs on the market. It is based on the detection of bacteria cell lesions after a short incubation time with the drugs [12]. It has been used especially in critical situations like sepsis, but here we describe its first application directly in positive urine samples.

UF-1000i is a urine flow cytometer from Sysmex Corporation (Kobe, Japan) (Sysmex®), which uses a diode laser to quantify sediment in two analytic channels and a fluorescent dye, which stains DNA. One channel analyzes only the microbial contents of the urine, while the other analyzes RBCs, WBCs, casts, and other non-microbial sediment. The staining agent is a fluorescent polymethine dye that binds to DNA. After staining, the particles are transported to a flow cell and are irradiated by a semiconducting laser (λ 635 nm) [13]. Its use to screen urine and select infected samples showed a sensitivity/specificity of 81.8/91.1% in detecting Gram-negative bacteria [14], allowing for the first example a rapid AST, where culturing was no longer performed.

A rapid AST requires the rapid identification of microorganisms (ID). MALDI-TOF technology, with its rapid ability to ID, allows for the identification of microorganisms from colonies. Excellent ID results were obtained when using the sample prep described on FASTinov kits directly from positive blood cultures, avoiding sub-culturing [15].

Preliminary data obtained using clinical samples from patients with UTIs and urine samples inoculated with well-known characterized bacteria, screened positive for GNB using the Sysmex 1000i system, are presented in this article regarding the direct ID of bacteria and the determination of the susceptibility to colistin via the FASTcolistin MIC in less than 2 h.

2. Results

MALDI-TOF correctly identified 100% of the samples in at least one of the spots. Table 1 shows the distributions of species in clinical and inoculated samples and the intervals of MIC values obtained by microdilution in the studied strains.

Table 1. Distribution of bacterial species analyzed on the FASTcolistin MIC, including patient samples and inoculated samples in urine; interval of minimal inhibitory concentrations (MIC—mg/L) obtained per species by microdilution.

	Species	Patients	Inoculated	Total	MIC
Enterobacterales	<i>Escherichia coli</i>	23	7	30	0.125->64
	<i>Klebsiella pneumoniae</i>	13	6	19	0.5-2
	<i>Klebsiella aerogenes</i>	2	1	3	1-2
	<i>Serratia marcescens</i>		1	1	8
	<i>Proteus mirabilis</i>	1	2	3	>64
	<i>Providencia rettgeri</i>		1	1	4
	Total	39	18	57	
No fermenters	<i>Pseudomonas aeruginosa</i>		30	30	0.25-4
	<i>Acinetobacter baumannii</i>		12	12	1-4
	<i>Enterobacter cloacae</i>		1	1	2
	Total	0	43	43	
Total studied		39	61	100	

Flow cytometry analysis of each strain was carried out in 20 min instrument time and the result was immediately obtained using the proprietary bioFAST software. Categorization of the phenotype and MIC value was performed for each strain and compared with microdilution. An example of cytometry histograms of a susceptible strain is presented in Figure 1. In the case of a resistant phenotype, the histogram is similar to that of the control, with at least colistin concentration of 4 mg/L, (over the 2 mg/L breakpoint concentration of both the EUCAST and CLSI protocols) [16,17]. Ten strains were resistant (MIC>2 mg/L) and all correctly detected by the FASTcolistin MIC kit.

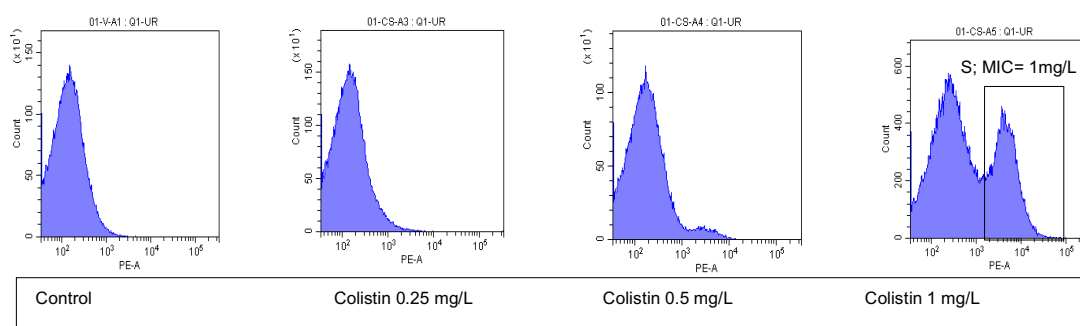


Figure 1. Flow cytometry histograms of an example of a susceptible (S) strain after exposure to different concentrations of colistin ranging from 0.25 mg/L to 1 mg/L; the control is the histogram of cells not exposed to the drug; all the cells represented were exposed to the fluorescent probe. After 1 mg/L of colistin, the histogram shows a drift of the population to the right, meaning that cell membrane lesions were forming as colistin was being effective (MIC value).

The essential agreement between the FASTcolistin MIC and reference methods was 99%, with a bias of -17% (Table 2).

Table 2. Correlation between the determination of the minimum inhibitory concentration (mg/L) via the reference method (microdilution) and flow cytometry (FASTcolistin MIC). MICs within essential agreement (within +/- 1 dilution of reference MIC) are highlighted in light grey and MIC identical in both tests are in dark grey. EUCAST and CLSI breakpoints are shown as lines. MIC ≤ 2 mg/l means Susceptible for EUCAST and Intermediate for CLSI; MIC ≥ 4 is resistant for both EUCAST and CLSI protocols.

	Reference method MIC (mg/L)(microdilution)									
	0.125	0.25	0.5	1	2	4	8	16	32	>64
FASTcolistin MIC (mg/L)	0.25	1	2	3						
	0.5		4	8	9					
	1		1	14	10	6				
	2				15	17				
	4						5			
	8							1		
	16									
	32									
	≥ 64									4

3. Discussion

UTIs, primarily caused by Gram-negative bacteria, pose a significant public health challenge due to their prevalence and risk of life-threatening sepsis. Patients with recurrent UTIs are submitted to several antimicrobial treatments per year, meaning they often develop multidrug-resistant strains with no good therapeutic options. Rapid and accurate diagnostic techniques are essential to guide targeted therapy, reducing the reliance on broad-spectrum antibiotics, which contribute to antimicrobial resistance.

A standard AST from urine is time-consuming, as it requires first sub-culturing for 24 h before identification and the AST can be performed. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) performed from colonies provides an immediate and accurate ID one day before an AST. It typically takes 2 days to complete a microbiology report, often resulting in limited and delayed clinical benefit. Syxmex developed a method for the screening analysis of urine that removes the need to smear negative samples, reducing the technical work required, as they are the majority. Clinical samples used in this study, screened positive with UF-1000i for GNB $>105/\text{mL}$, were used to ID and AST directly from urine after the FASTinov sample prep. The FASTcolistin MIC presented in this study offers a rapid, accurate method that could serve as an alternative to traditional AST methods, with a significant reduction in the time-to-result to under 2 h. This approach could substantially impact UTI management, reducing the time-to-result and time to therapy, which will be reflected in patient

outcomes. Patients put forward for colistin treatment are always critical patients without a lot of therapeutic options. The rapid detection of resistance will avoid therapeutic failure with a high risk of mortality.

Among the limitations of this study are the possibility that urine samples may be polymicrobial. Despite the Sysmex system and MALDI TOF detecting more than one population, polymicrobial samples may challenge them when we work directly with clinical samples. The other concern is the need for the method to be performed in a lab and with a few manual steps, although those are simple and could be performed for any lab technician.

For now, the Fastinov Colistin kit is only optimized for colistin E; in the future, it should be extended to other drugs and polymyxins, such as colistin B, widely used in the USA. Recently, methodologies for rapid ASTs in urine, like the PA-100 AST System, have been described. They include the susceptibility to only five commonly prescribed antibiotics for the treatment of uncomplicated UTIs: amoxicillin/clavulanic acid, ciprofloxacin, fosfomycin, nitrofurantoin, and trimethoprim. However, they can be performed easily in outpatient settings, while FASTinov technology, at the moment, can only be used in a clinical lab.

Further multicenter validation studies with FASTcolistin MIC testing in clinical urine samples are ongoing, as well as the expansion of the application of this technology to other antimicrobials (beta-lactamics, quinolones, aminoglycosides, fosfomycin, and nitrofurantoin), similar to the process followed with positive blood cultures [18].

In this work, financial aspects were not considered as this kit is not yet on the market [19].

4. Material and Methods

4.1. Urine Samples

Patients were recruited from the Urology Department of a tertiary center, after being admitted with UTIs. Ninety urine samples collected in sterile VACUETTE® tubes were analyzed on the UF-1000i system (Sysmex, Kobe, Japan), and thirty-nine positive for GNB ($>10^5$ /mL) were included in this study. Samples with Gram-positive microorganisms/contamination or with $<10^5$ /mL were excluded. Sixty-one urine samples collected from healthy volunteers were filtered with a 22 µm filter (Q-Max®, Frilabo, Maia, Portugal) and each one inoculated with characterized GNB strains from the cryobanked collection of the Microbiology Department, Porto Medical School (Portugal) before being incubated overnight. Two ATCC strains were included: *E. coli* 8739 with an MIC to

colistin of 0.5 mg/L, and *E. coli* 13846, positive for *mcr1* with an MIC to colistin >64 mg/L. The workflow is presented in Figure 2.

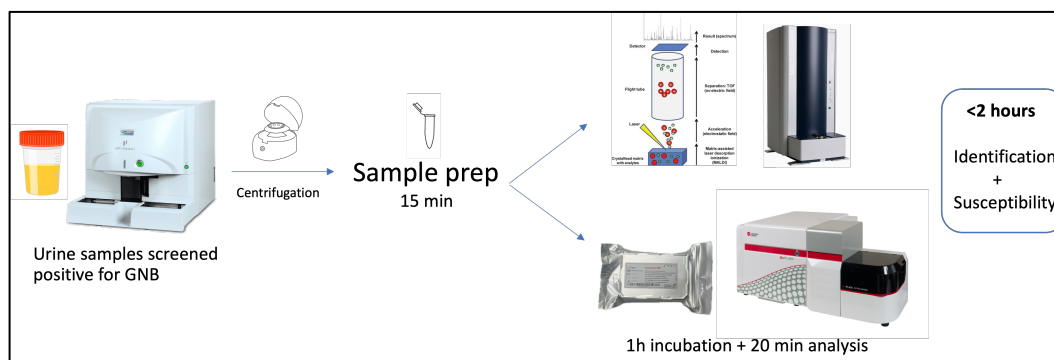


Figure 2. Workflow of this study performed with urine samples.

4.2. Sample Preparation and Identification

After centrifugation, 10 mL of urine sample was centrifuged at 3000 rpm for 10 min, supernatant was rejected, and the pellet was resuspended in 1 mL of sterile saline solution. Then, 50 μ L of hemolytic agent (tergitol 10%) was added and vortexed for 10s. The tube was centrifuged at 13,000 rpm for 1min; the supernatant was then rejected and the pellet resuspended in 500 μ L of saline solution. This suspension was carefully poured on top of 500 μ L of Histopaque[®] (Sigma-Aldrich, St. Louis, MO, EUA) (a density gradient medium) and centrifuged again at the same speed and for the same time. This achieved the removal of the remaining urine cells and debris, preserving bacteria. Two spots of pellets were inoculated in the Bruker MALDI Biotyper CA System (Bruker Daltonics, Billerica, MA, USA), and we performed an ID in a similar way to that for a colony.

4.3. Incubation with Colistin and Fluorescent Probe

A bacterial pellet was resuspended in 500 μ L of sterile saline solution, adjusted to 0.5 McFarland suspension, and diluted (1 mL in 7 mL) with Muller–Hinton cation-adjusted broth medium (Lioflchem, Ref.26177, Roseto degli Abruzzi, TE, Itália). The FASTcolistin MIC panel (96-well panel) from FASTinov[®] containing 9 concentrations of colistin dilutions, from the 3rd to the 11th wells, halved each time (0.25–64 mg/L), and a fluorescent probe (patent protected) was inoculated. Three controls were included: autofluorescence—1st well without drug or fluorescence probe; viability control—2nd well without drug but with fluorescence probe; and a dead control—12th well with benzydamine and the fluorescence probe, in order to be sure that the probe is active. Incubation was performed for 1 h at 35 ± 2 °C with shaking (550 rpm/min).

4.4. Flow Cytometry Analysis

Flow cytometry was performed using a CytoFLEX cytometer equipped with one blue laser (488 nm; output, 50 mW; beam spot size, 5 by 80 μm). The instrument has three fluorescence channels: 525/40 BP, 585/42 BP, and 690/50 BP. It is also equipped with a plate reader for the automatic analysis of each panel; each sample takes 15 min instrument time. Flow cytometry is similar to a fluorescence microscope but can analyze a significant number of cells in a few seconds, recording their size, complexity, and fluorescence intensity. Using optimized fluorochromes, we are able to see the damage that is being caused by drugs to bacteria after a short incubation time. As the bacteria are being “killed”, the population seen in the cytometry forward scatter line is moved to the right. This is noticeable in histograms. Although this change in cells is quite visual, Fastinov has software with its own algorithm, which interprets the changes and compares them to a large database, interpreting the results as susceptibility or resistance and guiding the report it produces.

The flow cytometer was used in the slow mode, and data analysis was automated using bioFAST software, a FASTinov proprietary software, which provided the MIC value and susceptibility category based on EUCAST and CLSI standards. A multiparametric analysis compared the treated cells with the non-treated cells (control of viability) regarding the sizes and complexity of the cells and the intensity of fluorescence, especially at 690/50 BP (PE-E).

4.5. Reference Method for Colistin Susceptibility

In parallel, all urine samples analyzed were sub-cultured, colonies identified using MALDI-TOF technology, and colistin susceptibility tested using the standard broth microdilution method, with the results interpreted according to the EUCAST/CLSI guidelines [16,19]. Briefly, a serial two-fold dilution of colistin was prepared in Muller–Hinton broth (MHB), and 100 μL of each colistin dilution was dispensed to the corresponding wells in a 96-well plate, with concentrations ranging from 0.25 mg/L to 64 mg/L. A standardized bacterial suspension was prepared by adjusting the turbidity of the inoculum to 0.5 McFarland standard (approximately $1\text{--}2 \times 10^8$ CFU/mL) and diluting the suspension in MHB to achieve a final inoculum concentration of 5×10^5 CFU/mL in each well. The bacterial inoculum was added to each well (100 μL) containing colistin, bringing the final volume in each well to 200 μL . The microplate was incubated for 24 h at 35 ± 2 $^{\circ}\text{C}$, and we visually check each well for bacterial growth by observing turbidity. The MIC was defined as the lowest concentration of colistin that completely inhibited visible bacterial growth.

4.6. Statistical Analysis

The essential agreement (EA) and bias of the FASTcolistin MIC were calculated according to International Organization for Standardization [20] taking microdilution as the standard.

5. Conclusions

The high frequency of recurrent UTIs, combined with the increasing prevalence of antimicrobial resistance, poses a substantial public health challenge. Urgent measures are needed to promote antibiotic stewardship and enhance diagnostic tools, which could help with patient treatment and prevent AMR. The FASTcolistin MIC kit is the fastest in the world when seeking to accurately determine the MIC directly from a positive urine sample.

Author Contributions: All authors contributed significantly to this work. Daniela Fonseca-Silva and R. G. conducted the experiments, analyzed the data and wrote the manuscript. I. M.-O. and A. S.-D. conducted the experiments. M. H. R. contributed to the discussion and manuscript preparation. C. P.-V. designed this study and collaborated on the discussion and writing. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study

Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: Rosário Gomes, Ana S. Dias, and Inês M. Oliveira are employees of FASTinov, S.A. Cidália Pina-Vaz is a co-founder of FASTinov, S.A. The remaining authors declare that this research was conducted in the absence of any commercial relationships that could be construed as potential conflicts of interest. FASTinov provided the kits and the training, though all the experiments were performed by Daniela Fonseca-Silva in a clinical and university hospital.

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Discussion and Future Perspectives

Colistin is often considered a last-resort treatment for patients infected with multi-resistant Gram-negative bacteria. Therefore, a rapid and reliable method for determining bacterial susceptibility to colistin is essential [1]. According to EUCAST and CLSI, broth microdilution (BMD) remains the only validated method for colistin susceptibility testing [2,3]. This is largely due to the physicochemical characteristics of colistin—a polymyxin that tends to bind to plastic surfaces—leading to inaccurate results in methods such as disk diffusion and E-tests [3]. Furthermore, several studies have shown that some automated susceptibility testing systems produce also unreliable results [2].

Despite being the gold standard, BMD is labor-intensive and results are only available 48 to 72 hours after sample collection [3,4]. Patients requiring colistin are often critically ill, having failed first-line treatments, highlighting the need for faster yet reliable diagnostic methods [1,4].

Flow cytometry has shown excellent results as a rapid phenotypic method directly from blood culture or colonies. Although regarding colonies the determination of the susceptibility to many antibiotics, such as beta-lactams, requires the bacterial incubation in broth (2-3 h) since these drugs act only on bacteria at exponential growth phase [5,6]. However, colistin targets the bacterial membrane directly, allowing the use of colonies directly. A total saving of more than 22 hours was observed.

Avoiding the time for sub-culturing excellent correlation has been demonstrated between flow cytometry results and the reference method when using positive blood cultures [5,7]. The same was observed with the test of colistin directly from blood culture saving around 2 days to give the results.

In the initial development of this method regarding the determination of susceptibility to colistin directly from blood cultures, a breakpoint test (qualitative) was developed as described in the published article; only 2 concentrations of colistin (2 and 4 ug/ml) were tested. Later, the study was expanded to include MIC determination (quantitative results), which was presented as an oral communication at ECCMID 2023 (ANNEX I) Breakpoint test using flow cytometry is even more rapid but MIC determination will allow future adjustments if breakpoint thresholds are revised. The FASTcolistinMIC, now a commercial test is a quantitative and a qualitative assay. Because the cytometer reads each well sequentially, using breakpoints instead of full MIC determination can speed up reporting, especially when multiple antibiotics are tested in parallel like in other FASTinov® panels. However, for special patients often with many co-morbidities, determining the exact MIC may be useful on the therapeutic management of the patient,

allowing dose spacing or adjustment. Still, in many clinical cases, the categorical result (susceptible/resistant) is sufficient for the majority of the patients.

Regarding bacteria incubation time with colistin, initial experiments demonstrated that 15 minutes was sufficient for detecting membrane damage via flow cytometry using the selected fluorochrome. However, this time was later extended to 60 minutes, as certain bacterial strains showed less consistent correlation with MIC values at the shorter timepoint.

As the study evolved, MIC determination was extended not only to colonies but also to blood cultures and urine samples, ensuring consistent results across different specimen types.

Colistin is important on the treatment of urinary tract infections (UTIs), especially those that are recurrent or difficult to treat. With the same sample preparation that we used for blood cultures, urine were directly inoculated into the FASTcolistin MIC gallery, bypassing the need for subculturing saving almost 2 days [6].

The method is easy to perform but the analysis requires a flow cytometer equipped with a microplate reader—an instrument that is not available in most clinical microbiology laboratories and represents a significant financial investment [5].

This assay is currently semi-automated, not yet fully automated, which may limit its adoption and scalability in routine diagnostics.

Another practical limitation is that many clinical microbiologists are not yet familiar or fully comfortable with flow cytometry, which may pose a barrier to its widespread adoption in microbiology laboratories.

Phenotypic assays offer safer guidance for antimicrobial therapy than molecular methods. Although the presence of the *mcr-1* gene confirms resistance to colistin, its absence does not guarantee susceptibility, due to the existence of other genes conferring resistance [8]. Recently techniques such as MALDI-TOF MS, through the analysis of membrane lipids, were described with the ability to rapidly identify microorganisms presenting *mcr-1*-mediated resistance without the need of culture. However, this method is specific to a single resistance mechanism, necessitating confirmation of negative results by alternative approaches [9].

Another important technical adjustment was the choice of fluorescent stain: propidium iodide (PI) proved to be more effective than DiBAC when assessing colistin-induced damage, due to the drug's unique mechanism of action on the bacterial membrane. As

a result, colistin was separated from the standard Gram-negative antibiotic panel used in the FASTinov kit and is now tested independently for MIC determination. Initially, it was included in the broader panel to support validation, but the specificity and performance of PI justified a dedicated test.

The FASTcolistin platform could potentially expand to include polymyxin B, which is less nephrotoxic and more widely used in the United States [10]. Additionally, similar MIC galleries should be developed for other last-line antibiotics such as cefiderocol, meropenem-vaborbactam, and imipenem-relebactam [10].

Flow cytometry could also be used to test synergy between drugs so important in critical and difficult clinical situation. Classic methodologies for synergic study are often impractical due to its complexity and time requirements. Flow cytometry-based synergy tests could offer a viable alternative, especially since combination therapy is frequently used without *in vitro* validation of its efficacy [5,6].

To further optimize treatment and reduce toxicity, therapeutic drug monitoring (TDM) is essential. Current TDM methods for antibiotics, such as high-performance liquid chromatography (HPLC) and immunoassays, are chemically based, expensive, and limited to a few drugs [11]. Flow cytometry has the potential to quantify the biologically active fraction of colistin directly in clinical samples, such as blood [11].

Preliminary data have been produced supporting the development of a flow cytometry-based TDM assay capable of evaluating colistin activity in under two hours. A patent was meanwhile filled and the validation of the method in critically ill patients, including those undergoing extracorporeal membrane oxygenation (ECMO), dialysis, or presenting with severe edema will take place [11]. During the time of developing this thesis there was no opportunity to initiate that assay but a clinical validation study will be designed.

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CHAPTER 7

Final Remarks

Concluding, this work, can represent an alternative to the reference method for colistin evaluation of the susceptibility.

Although some companies try to establish new methods for determining MICs, European guidelines for microbiology laboratories (EUCAST – European Committee on antimicrobial susceptibility testing) recently argue that tests have errors and must be recalibrated for the correct determination of the concentration gradient. They refer that the currently available gradient tests should be withdrawn from the laboratory until further notice.

This research might lead to the establishment of a new susceptibility test protocol for colistin and contribute to a faster response of microbiology laboratories, thus allowing early target therapy.

Given the obtained results, this new and fast flow cytometric assay demonstrated an excellent tool for evaluation of colistin activity providing a MIC value in useful time, showing a high agreement with the reference method (broth microdilution) and a reduced time to results (TTR) (± 3 hours versus 48h required for current methodologies) directly from culture plate as well as from BC samples.

This methodology may represent an alternative to reference method, difficult to perform in clinical labs.

- Abstracts: Oral Communications and Posters
- Guest Speaker Certificates
- Awards Certificates

ABSTRACTS: ORAL COMMUNICATIONS AND POSTERS

- “A novel flow cytometric protocol for the evaluation of colistin activity”, Daniela Fonseca e Silva, R. Teixeira-Santos, A. Dias, S. Oliveira, I. Oliveira, M H Ramos, A Gonçalves Rodrigues, C Pina-Vaz; 10ª Reunião Científica da Sociedade Portuguesa de Medicina Laboratorial, April 13 and 14, 2018; Figueira da Foz – **Oral Communication**.



ABSTRACT

Background: Colistin (CS) constitutes the last resort for infections caused by multidrug-resistant Gram-negative bacteria.^{1,2} Classical susceptibility tests are laborious, time-consuming and some technical issues have been reported.³

Aims: Optimization of a novel flow cytometric protocol to evaluate qualitative and quantitatively cell physiological changes induced by CS exposure.

Methods: Were included 2 susceptible (*P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922) and 1 resistant strain to CS [*E. coli* NCTC 13846 (*mcr-1*-positive, MIC 8 µg/mL)].

Bacteria were inoculated in Muller-Hinton broth (Sigma) until exponential growth phase (± 2 hours). A cell suspension containing 1×10^6 cells/mL was incubated during 15, 30 and 60 min, at 37°C with CS (C4461, Sigma) at concentrations ranging between 0.25 to 8 $\mu\text{g/mL}$, and stained with different fluorescent dyes. Changes in membrane lesion, membrane potential, metabolic activity and ROS production were explored. Afterwards, cells were analyzed in a BD Accuri™ C6 Plus Flow Cytometer. For each tested conditions the intensity of fluorescence of treated cells and non-treated cells were compared determining a staining index. Additionally, this protocol was applied in pure colonies directly from culture plate. Different statistical tests were used (Wilcoxon and Kruskal-Wallis tests). P -value < 0.05 was considered significant.

Results: CS induced cellular alterations in susceptibility bacteria, regarding membrane lesion, membrane potential, metabolic activity and ROS production up to 1 hour of exposure. 15 min were sufficient to produce changes in membrane lesion, being this effect dose-dependent. Conversely, in R strain, the studied cellular parameters did not suffer significant changes up to 60 min of incubation. Significant differences were found between S and R strains ($p < 0.001$). Data obtained for the protocol with pure colonies directly from culture plate were similar to described above, with the advantage of providing a result in lower time. Moreover, cytometric data showed a high agreement (1.00) with the MIC values of CS previously determined according to CLSI and EUCAST protocols.

Conclusions: We hereby presented a new and fast flow cytometric protocol which revealed to be an excellent tool for evaluation of CS activity providing a MIC value in useful time.

References:

- ¹Loho T, et al.; *Colistin: an antibiotic and its role in multiresistant Gram-negative infections*; Acta Med Indones (2015 Apr); 47(2):157-168.
- ²Lim L, et al.; *Resurgence of Colistin: A Review of Resistance, Toxicity, Pharmacodynamics and Dosing*; Pharmacotherapy (2010 Dec); 30(12): 1279-1291.
- ³Richter SS, et al.; *Evaluation of Sensititre Broth Microdilution Plate for determining the susceptibility of carbapenem-resistant Klebsiella pneumoniae to polymyxins*; Diagn Microbiol Infect Dis (2018 Jan), doi: 10.1016/j.diagmicrobio.2018.01.005.

- **“Optimization of a novel flow cytometric protocol for the study of colistin activity”** Daniela Fonseca e Silva, R. Teixeira-Santos, A. Dias, S. Oliveira, I. Oliveira, M H Ramos, A Gonçalves Rodrigues, C Pina-Vaz; 28th ECCMID; April 21 to 24, 2018; Madrid, Espanha – **Oral communication.**



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14 May 2018

To whom it may concern:

CONFIRMATION OF PRESENTATION AT ECCMID 2018

We hereby confirm that the following abstract has been submitted, accepted and presented at the 28th ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases, which took place in Madrid, Spain, 21 – 24 April 2018.

Title: Optimization of a novel flow cytometric protocol for the study of colistin activity

Abstract Authors: D. Fonseca e Silva, R. Teixeira-Santos, A. Silva-Dias, S. Costa-de-Oliveira, I. Martins-Oliveira, M. Ramos, A. Rodrigues, C. Pina-Vaz

Presenter: Daniela Fonseca e Silva

Session Title: Colistin antimicrobial susceptibility testing - which methods are available?

Presentation Type: 2-Hour Oral Sessions With Mini-Review

Presentation Number: O0956

Yours sincerely,

Winfried V. Kern
ECCMID 2018 Programme Director

ESCMID European Society of Clinical Microbiology and Infectious Diseases

ESCMID Executive Committee M. Poljak, President, Ljubljana, SI; M. Akova, Past-President and Communication Officer, Ankara, TR; J. Rodriguez-Balio, President-elect and Secretary General, Seville, ES; A. Friedrich, Treasurer, Groningen, NL; M. Sangalli, Professional Affairs Officer Clinical Microbiology and Infectious Diseases, Rome, IT; E. Tacconelli, Education Officer, Tübingen, DE; A. Zinkarnagel, Scientific Affairs Officer, Zurich, CH

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28th ECCMID
EUROPEAN CONGRESS OF
CLINICAL MICROBIOLOGY
AND INFECTIOUS DISEASES
Madrid, Spain
21 – 24 April 2018

ABSTRACT

Background: Colistin (CS) is being increasingly used as a “last-line” option to treat infections caused by multidrug-resistant Gram-negative bacteria. Classical susceptibility tests takes a long time-to-result (24-48h). Additionally, problems in performing *in vitro* susceptibility of CS were reported. The present study aims the optimization of a novel flow cytometric protocol to evaluate qualitative and quantitatively cell lesions induced by CS in clinical useful time.

Materials/methods: Two susceptible (*P. aeruginosa* ATCC 27853 and *E.coli* ATCC 25922) and one resistant strain to CS [*E. coli* NCTC 13846 (*mcr-1*-positive)], were

studied. Bacteria were incubated during 15, 30 and 60 min, at 37°C with CS (C4461, Sigma) at concentrations ranging between 0.25 to 8 µg/mL, and stained with different fluorescent dyes. Membrane lesion, membrane potential, metabolic activity and ROS production were investigated. Cells were then analyzed in a BD Accuri™ C6 Plus Flow Cytometer. For each tested conditions the intensity of fluorescence of treated cells were compared with non-treated cells. The difference between the distinct times of incubation were evaluated using Wilcoxon test. For the comparison of the cytometric profile between S and R strains the Kruskal-Wallis test was used. *P*-value <0.05 was considered significant.

Results: Susceptible bacteria treated with CS displayed cellular alterations, namely membrane lesion, impairment membrane permeability, metabolic activity, and ROS production. In R strain, the studied cellular parameters did not suffer significant changes up to 60 min of incubation. Fifteen minutes were sufficient to produce changes in membrane lesion, and this effect revealed to be dose-dependent; significant differences were found between S and R strains at this time point ($p < 0.001$). Moreover, cytometric data showed a high agreement (1.00) with the MIC values of CS previously determined according to CLSI and EUCAST protocols (EN ISO 20776-1, 2016), specially using a membrane lesion stain.

Conclusions: We hereby propose a new and fast method for colistin susceptibility testing, providing a MIC value in a clinical useful time, thus allowing early target therapy.

- “Finding a novel flow cytometry protocol for the evaluation of colistin susceptibility directly from blood cultures”, Daniela Fonseca e Silva, R. Teixeira-Santos, A. Dias, S. Oliveira, I. Oliveira, M H Ramos, A Gonçalves Rodrigues, C Pina-Vaz; ASM Microbe, June 7 to 11, 2018; Atlanta, Georgia, United States of America.
- Poster

ABSTRACT

Background: The increasing prevalence of multidrug-resistant Gram-negative bacteria worldwide leads to a re-evaluation of the previously discarded antibiotic, such as Colistin (CS), belonging to the class of polymyxins, is being increasingly used as a ‘last-line’ therapy to treat infections caused by multidrug resistant (MDR). However, the increase in the use of CS has resulted in the emergence of clinical isolates with reduced susceptibility to this antibiotic. Classical susceptibility tests are laborious and time-consuming (minimum of 24h). Simultaneously, technical issues have been reported. The present study intend to develop a flow cytometry method that can easily and quickly analyze bacteria extracted directly from blood cultures (BC) samples and qualitative evaluate the susceptibility for CS.

Methods: A total of 25 BC were spiked with well characterized bacteria including recommended AST control strains (11 Enterobacteriaceae, 12 *Pseudomonas* spp. and 2 *Acinetobacter* spp.), inoculated with human blood and incubated until flag positive. Bacteria was extracted from BC, inoculated in a microplate containing colistin in variable concentrations and a fluorescent dye that detect cell membrane lesion; after incubation (1h), the susceptibility of colistin was analyzed by Accuri™ C6 Flow Cytometer. EUCAST and CLSI protocols were selected on the FASTinov dedicated software. In order to check the accuracy of FASTinov® gramneg for evaluation of colistin susceptibility, error rates [minor (mE), major (ME) and very major (VME)], categorical agreement (CA) and accuracy measurements were calculated.

Results: The time-to-result of the method applied was significantly shorter (2 h vs. a minimum of 24 h), potentially allowing an earlier therapy. No systematic errors were found either minor, major or very major rates. These results validate the method to be used in laboratory for measuring with a good precision and accuracy (sensitivity and specificity of 1.00). Moreover, cytometric data showed a high agreement (1.00) with susceptibility determined according to CLSI and EUCAST protocols (EN ISO 20776-1, 2016).

Conclusion: We hereby presented a new and fast flow cytometric protocol which revealed to be an excellent tool for evaluation of CS activity directly from BC samples.

- **“Evaluation of Colistin Susceptibility Directly from Blood Cultures – A Novel Flow Cytometric Protocol”, Daniela Fonseca e Silva, R. Teixeira-Santos, A. Dias, I. Oliveira, M H Ramos, A Gonçalves Rodrigues, C Pina-Vaz; European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE) 2018, November 21 to 23, 2018; Saint Julian’s, Malta - **Poster**.**



CERTIFICATE OF ATTENDANCE

This is to certify that Mrs Daniela Fonseca e Silva has attended the European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE) which was held in Saint Julian's, Malta from 21- 23 November 2018.
Mrs. Fonseca e Silva has attended the conference on 21 November and she gave a poster presentation with the title: *“Evaluation of Colistin Susceptibility Directly from Blood Cultures - A Novel Flow Cytometric Protocol”*.


Prof. Mike Catchpole
Chair of the ESCAIDE Scientific Committee





EVALUATION OF COLISTIN SUSCEPTIBILITY DIRECTLY FROM BLOOD CULTURES – A NOVEL FLOW CYTOMETRIC PROTOCOL

Daniela Fonseca e Silva^{1,2}, Rita Teixeira-Santos³, Ana Silva-Dias³, Sofia Costa-de-Oliveira^{1,4}, Inês Martins-Oliveira³, Maria Helena Ramos^{1,2}, Acácio Rodrigues^{3,4}, Cidália Pina-Vaz^{3,4}

¹ Clinical Microbiology Department, Porto Hospital and University Centre, Porto, Portugal; ² Abel Salazar Institute of Biomedical Sciences, University of Porto, Porto, Portugal; ³ FASTinov, SA; ⁴ Department of Microbiology, Faculty of Medicine of Porto University, Porto, Portugal

ABSTRACT

Colistin (CS), recently reintroduced in clinical practice, constitutes the last resort for treating infections caused by multidrug-resistant (MDR) bacteria.^{1,2}

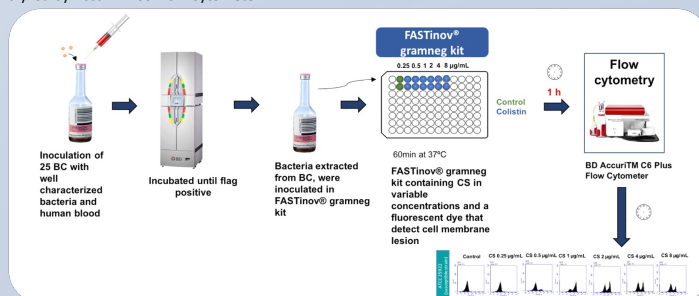
Classical susceptibility tests are laborious, time-consuming and technical issues have been reported.³

The present study intends to develop a flow cytometric method that can easily and quickly analyze bacteria extracted directly from blood cultures (BC) samples and qualitative evaluate the susceptibility for CS.

METHODS

25 BC were spiked with well characterized bacteria including recommended AST control strains (11 Enterobacteriaceae, 12 Pseudomonas spp. and 2 Acinetobacter spp.), inoculated with human blood and incubated until flag positive.

Bacteria extracted from BC, were inoculated in FASTinov® gramneg kit containing CS in variable concentrations and a fluorescent dye that detect cell membrane lesion; after incubation (1h), the susceptibility of CS was analyzed by AccuriTMC6 Flow Cytometer.



The comparison between the new protocol and susceptibility previous determined according to EUCAST and CLSI was performed. In order to check the accuracy of this new method, error rates [minor (mE), major (ME) and very major (VME)] and categorical agreement (CA) were calculated.

RESULTS

The time-to-result of flow cytometric protocol was significantly shorter than routine methodologies (2h vs. 24 h), potentially allowing an earlier therapy.

Cytometric data showed a high agreement (1.00) with susceptibility determined according to CLSI and EUCAST protocols.

No systematic errors were found either minor, major or very major rates.

These results validate the method to be used in laboratory for measuring with a good precision and accuracy (sensitivity and specificity of 1.00).

CONCLUSIONS

We hereby presented a new and fast flow cytometric protocol which revealed to be an excellent tool for evaluation of CS susceptibility directly from BC samples, with high agreement with broth microdilution reference method.

REFERENCES

- ¹Loho T, et al.; Colistin: an antibiotic and its role in multiresistant Gram-negative infections; Acta Med Indones (2015 Apr); 47(2):157-168.
- ²Lim L, et al.; Resurgence of Colistin: A Review of Resistance, Toxicity, Pharmacodynamics and Dosing; Pharmacotherapy (2010 Dec); 30(12): 1279-1291.
- ³Richter SS, et al.; Evaluation of Sensititre Broth Microdilution Plate for determining the susceptibility of carbapenem-resistant Klebsiella pneumoniae to polymyxins; Diagn Microbiol Infect Dis (2018 Jan), doi: 10.1016/j.diagmicrobio.2018.01.005.

- “**Rapid evaluation of the susceptibility profile of Gram-negative bacteria of colistin directly from blood cultures**”; Daniela Fonseca e Silva, R. Teixeira-Santos, A. Dias, I. Oliveira, M H Ramos, A Gonçalves Rodrigues, C Pina-Vaz; 29th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID); April, 13 and 14, 2019; Amsterdam, Holand - **Poster**.



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Basel, 9th May 2019

To whom it may concern:

CONFIRMATION OF PRESENTATION AT ECCMID 2019

We hereby confirm that the following abstract was submitted, accepted and presented at the 28th ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases, which took place in Amsterdam, Netherlands, 13 – 16 April 2019.

Title: Rapid evaluation of the susceptibility profile of Gram-negative bacteria to colistin directly from blood cultures

Abstract Authors: D. Fonseca e Silva, R. Teixeira-Santos, A. Silva-Dias, I. Martins-Oliveira, H. Ramos, A. Rodrigues, C. Pina-Vaz

Presenter: Daniela Fonseca e Silva

Session Title: Rapid phenotypic AST

Presentation Type: Paper Poster Session

Presentation Number: P2817

Yours sincerely,

Winfried V. Kern
ECCMID 2019 Programme Director

ESCMID European Society of Clinical Microbiology and Infectious Diseases

ESCMID Executive Committee: J. Rodriguez-Bard, President, Seville, ES; M. Poljak, Immediate Past-President and Publications Officer, Ljubljana, SI; M. Sengupta, President-elect and Secretary General, Rome, IT; A. Friedrich, Treasurer, Groningen, NL; E. Cambau, Professional Affairs Officer, Paris, FR; E. Tuccillo, Guidelines Officer, Verona, IT; A. Zelenberg, Scientific Affairs Officer, Zurich, CH; C. Espigul, Education and Communications Officer, Istanbul, TR; Ad hoc Members: Ch. Giske, EUCAST Chairperson, Stockholm, SE; L. Sordet, ESCMID Guidelines Director, Paris, FR; L. Leibson, CME Editor-in-Chief, Peppin-Town, IL; W. Kern, ECCMID Programme Director, Freiburg, DE

Amsterdam, Netherlands
13 – 16 April 2019
28th ECCMID
EUROPEAN CONGRESS OF
CLINICAL MICROBIOLOGY
AND INFECTIOUS DISEASES

ABSTRACT

Category: 3c. Susceptibility testing methods

7755 Rapid evaluation of the susceptibility profile of Gram-negative bacteria to colistin directly from blood cultures

Background: An accurate method for antimicrobial susceptibility testing (AST) of colistin is crucial in an era of increasing multi-resistance among Gram-negative bacteria and simultaneous increasing colistin resistance. Both EUCAST and CLSI recommend broth microdilution (BMD) for antimicrobial susceptibility testing of colistin, but BMD is rarely used in routine microbiology laboratories due to being cumbersome and providing late results. We have evaluated a fast antimicrobial susceptibility test developed by FASTinov, performed directly from positive blood cultures (BC).

Materials/methods: BC (BD) were spiked with 107 *Enterobacteriaceae*, 40 *Pseudomonas* spp and 26 *Acinetobacter baumannii*, inoculated with human donor blood and incubated until obtaining a positive flag; *E. coli* ATCC 13846 (mcr-1 gene positive) was included. Bacteria were extracted according with user instructions and incubated with colistin and the adequate fluorescent dye, during 1h at 37°C. Afterwards, the cells were analyzed on a CytoFLEX flow cytometer (Beckman Coulter). Results were automatically provided by BioFAST software, and compared with microdilution.

Results: The overall agreement between FASTinov cytometric test and reference method was 97,2% for *Enterobacteriaceae*, 95% for *Pseudomonas* spp and 100% for *Acinetobacter*. The highest major discrepancies were detected with *Pseudomonas* (2/31); the highest very major discrepancies were detected only with *Enterobacteriaceae* (2/16).

Conclusions: Our results support the accuracy on the FASTinov® cytometric test for assessment of antimicrobial susceptibility directly from positive blood cultures in a TTR of 2h (versus 48h required for current methodologies). This methodology is easier to perform in clinical labs and providing results in useful clinical time represents a real alternative.

- **“Rapid evaluation of the susceptibility of colistin directly from blood cultures”**; Daniela Fonseca e Silva, R. Teixeira-Santos, A. Dias, R. Gomes, I. Oliveira, M H Ramos, A Gonçalves Rodrigues, J. Palmeira, C Pina-Vaz; ASM Microbe, April 20 to 24, 2019; San Francisco, United States of America - **Poster**.



INTRODUCTION

An accurate method for antimicrobial susceptibility testing (AST) of colistin (CS) is crucial in an era of increasing multi-resistance Gram-negative bacteria and increasing colistin resistance. Blood culture (BC) represents the gold standard for identification (ID) and antimicrobial susceptibility testing (AST) of organisms causing bloodstream infections (BSI) or septicemia.

Both EUCAST and CLSI recommend broth microdilution (BMD) for antimicrobial susceptibility testing (AST) of colistin. However, BMD is rarely used in routine microbiology laboratories, as it is cumbersome and takes 24h to give results from pure colonies.

The currently reference procedure requires positive BCs to be sub-cultured on solid media to perform ID and AST by automated systems, thus requiring usually 48–72 h from BC positivity to AST results available to clinicians.

Hence, we have evaluated the performance of a fast antimicrobial susceptibility test developed by FASTinov® (Portugal), based on flow cytometry, that evaluate several phenotypic lesions induced by antimicrobial drugs, performed directly from positive BC.

MATERIALS/METHODS

Blood culture bottles (BD) were spiked with 170 gram-negative bacilli (107 Enterobacteriales, 31 *Pseudomonas* spp and 32 *Acinetobacter baumannii*), inoculated with human donor blood and incubated until a positive flag is obtained. A fully CS-susceptible *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, and the CS-resistant *E. coli* NCTC 13846 (CCUG 70662, DSM 105182; MIC of 4 µg/mL) were used as quality control. Bacteria were extracted from BC by centrifugation in a gel tube and inoculated with two concentrations of CS (2 and 4 µg/mL) and a fluorescent dye that shows membrane damage, during 1h incubation at 37°C. Afterwards, the cells were analyzed in parallel on CytoFLEX (Beckman Coulter) and Accuri (BD) flow cytometers. Results were automatically provided by BioFAST software, and compared with microdilution.

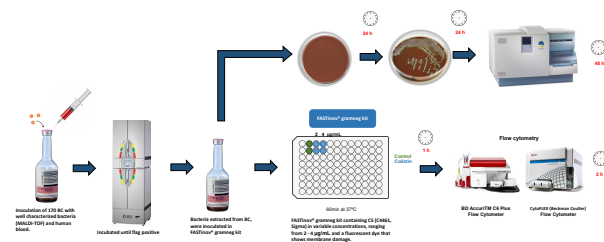


Figure 1. Schematic illustration of the workflow. BC (blood cultures); CS (colistin)

RESULTS

The overall agreement between FASTinov® cytometric test and reference method was 100% for Enterobacteriaceae and *Acinetobacter* spp. And 93,6% for *Pseudomonas* spp for both EUCAST and CLSI protocols (table 1). Regarding *Pseudomonas*, only one major and one very major error were detected, corresponding to a ± 2 dilutions apart from the breakpoint.

No differences were detected between the performance of the CytoFLEX and Accuri cytometers.

	EUCAST					CLSI				
	BMD	S	R	CA	ME	BMD	S	R	CA	ME
Gram-negative bacteria										
Enterobacteriales	107	91	16	100.0	-	91	16	100.0	-	-
<i>Pseudomonas</i> spp.	31	28	3	90.6	1 (3.5)	28	3	93.6	1 (3.5)	1 (3.5)
<i>Acinetobacter</i> spp.	32	31	1	100.0	-	31	1	100.0	-	-
Overall	170	150	20	88.8	1 (2.0)	150	20	88.8	1 (2.0)	1 (2.0)

Table 1 – Agreement rates in AST results for CS between the flow cytometric test and broth microdilution method.

CONCLUSIONS

Data support the accuracy on the FASTinov® cytometric test for determination of antimicrobial susceptibility directly from positive blood cultures in a TTR of 2h (versus 48h required for current methodologies). This new methodology may represent an alternative to the reference method, which is difficult to perform in clinical labs.

- “**Colistin MIC determination by a rapid flow cytometry assay**”; Daniela Fonseca e Silva, R. Gomes, I. Oliveira, A. Dias, M H Ramos, A Gonçalves Rodrigues, , C Pina-Vaz; 30th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), April 18 to 21, 2020; Paris, France. Poster number 8222 - **Poster**.



Basel, January 21, 2020

Dear Daniela Fonseca e Silva,

Abstract number: 8222

Title: **Colistin** MIC determination by a rapid flow cytometry assay

Thank you for having submitted your work to **ECCMID**, 18 – 21 April 2020, Paris, France. The **ECCMID 2020** Programme Committee is pleased to inform you that the above-mentioned abstract has been allocated to the following session type:

Session type:	Mini-oral ePoster Session
Session name:	Novel methods for RAST of XDR Gram-negatives
Presenting author:	Daniela Fonseca e Silva

The session details will be available on www.eccmidlive.org from 30 January 2020.

Please find all technical information regarding your session on our [website](http://www.eccmidlive.org). Presenting Author changes must be emailed to eccmidabstracts@eccmid.org. Kindly note that only authors of the abstracts can become presenting author. The author list cannot be changed anymore, however removing authors is possible. Please contact the office for help.

For those of you, who have a presentation in an oral session: Please note that you are required to list any disclosures in the first slide of your presentation.

The final scientific programme will be available online on www.eccmidlive.org and gets updated constantly; the abstracts bodies will be released 08 April 2020.

ABSTRACT

Background: The increasing prevalence of multidrug-resistant Gram-negative bacteria worldwide leads to a re-evaluation of previously discarded antibiotics, such as colistin (CS), belonging to the class of polymyxins. Its use is being increasingly as a ‘last-line’ resort therapy. The emergence of clinical isolates with reduced susceptibility to this antibiotic has been reported. As susceptibility results obtained with automated systems are not valid due to technical issues, different minimum inhibitory concentration (MIC) assays appear in the market. Nevertheless, they are laborious and time-consuming (minimum of 24h). In present study we evaluate a rapid flow cytometry method that can easily and quickly determine CS MIC values in 2 hours versus 24h from pure colonies with standard broth microdilution.

Materials/methods: A total of 84 *Enterobacterales* isolates, including 12 QC control strains, with well characterized mechanisms were tested for MIC determination by flow cytometry and compared with broth microdilution (EN ISO 20776-1, 2016) interpreted according to CLSI and EUCAST criteria protocols. For flow cytometry assay (FC), bacteria were inoculated in a microplate containing colistin in variable concentrations (from 0-25 to 32 mg/L) together with a fluorescent dye that detect cell membrane lesion; after incubation (1h), the susceptibility of colistin was analyzed by CytoFLEX Flow Cytometer. FASTinov results were provided by a dedicated software. Categorical agreement (CA) and Essential agreement (EA) were calculated.

Results: The distribution of the strains by CS MIC are represented in Table1. The CA was 100% and the EA 90.5% with 6 strains showing by FC MIC values 2 dilutions below the reference method and 2 strains 2 dilutions above.

Table 1. Distribution of strains by colistin MIC

MIC values (ug/ml)	<0.125	0.25	0.5	1	2	4	8	16	>32	Total
Number of strains	11	10	20	9	2	9	5	3	15	84

Conclusions: Colistin induced cellular alterations when challenged with bacteria, regarding membrane lesion up to 1h of exposure, which allowed the determination of MIC value with high accuracy.

- “Determinación rápida directa de la sensibilidad a colistina mediante citometría de flujo en muestras de orinas”; Daniela Fonseca e Silva, Rosário Gomes, Blanca Pérez-Viso, Sara Cruz, Inês Martins-Oliveira, Ana Silva Dias, Maria Helena Ramos, Rafael Cantón, Acácio G. Rodrigues, Cidália Pina-Vaz. XXV SEIMC, June 2 to 4, 2022; Granada, Spain – **Oral communication**.



CERTIFICADO DE PARTICIPACIÓN

La Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica – SEIMC

CERTIFICA QUE:

D. Silva, R. Gomes, B. Pérez Viso, I. Martins Oliveira, A. Dias, A. G Rodrigues, R. Cantón, C. Pina Vaz

Han realizado la presentación **ORAL** de la comunicación con título:

Determinación rápida y directa de la sensibilidad a colistina mediante citometría de flujo en muestras de orinas positivas

En el **XXV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica** celebrado en Granada del 2 al 4 de junio del 2022.

Granada, a 4 de junio de 2022

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Dr. Antonio Rivero
Presidente de la SEIMC

Dra. Carmen Fariñas
Presidenta Comité Científico Congreso

0054

ABSTRACT

TRABAJOS ACEPTADOS	
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Enlace publicación	https://intranet.pacifico-meetings.com/amsysweb/faces/publicacionOnlineLIBRO.xhtml?id=749
Tema	09. Métodos fenotípicos y moleculares de diagnóstico en microbiología y de determinación de la sensibilidad a los antimicrobianos
Sesión	SO-06. Métodos fenotípicos y moleculares de diagnóstico en microbiología y de determinación de la sensibilidad a los antimicrobianos I
Código de presentación	0054
Autor(es)	Daniela Silva ¹ , Rosario Gomes ² , Blanca Pérez Viso ³ , Inês Martins Oliveira ² , Ana Dias ² , Acacio G Rodrigues ⁴ , Rafael Cantón ³ , Cidália Pina Vaz ²
Centros	¹ Instituto Português de Oncologia Porto Francisco Gentil, Oporto, ² FASTinov, Oporto, ³ Servicio de Microbiología Hospital Ramón y Cajal, Madrid, ⁴ CINTESIS-Center for Health Technology and Services Research, Faculty of Medicine of the University of Porto, Portugal., Oporto
Título:	Determinación rápida y directa de la sensibilidad a colistina mediante citometría de flujo en muestras de orinas positivas

Texto:

Introducción/Objetivos: Las infecciones del tracto urinario por bacilos gramnegativos (BGN) son muy frecuentes y la creciente aparición de multirresistencias compromete su tratamiento terapéutico. Colistina es utilizado en pacientes críticos como terapia de última línea y conocer su sensibilidad es necesario; a este problema se suma la dificultad técnica de su determinación *in vitro*. En este trabajo se ha utilizado la citometría de flujo como técnica rápida para determinar la sensibilidad a colistina directamente de muestras de orinas positivas.

Material y métodos: Para la realización de este estudio se utilizaron 114 muestras de orinas positivas en BGN (>10⁵/m) mediante el sistema automatizado UF-1000i (Syxmex®); 49 pertenecientes a pacientes y 65 inoculadas con BGN previamente caracterizados. Para la determinación de la sensibilidad a colistina se empleó la tecnología FASTinov® mediante el kit FASTcolistin MIC, compuesto de una microplaca que incorpora colistina a diferentes concentraciones, un fluorocromo capaz de mostrar las lesiones celulares provocadas por éste antibiótico y el software, BioFAST que proporciona el valor de la CMI. Los resultados se compararon con el método de referencia (microdilución). Se centrifugaron 5ml de orina (5000 rpm/10min) y el pellet se resuspendió en 1ml de salino estéril. Después se utilizó un agente hemolítico + Histopaque® (Sigma-Aldrich) para purificar la muestra y se centrifugó (13.000 rpm/1min); el pellet obtenido se resuspendió en 1ml de salino y la suspensión bacteriana se ajustó a 0.5 McFarland para la inoculación del kit FASTinov®. Las placas se incubaron a 37°C durante 1h y para el análisis se utilizó el citómetro Cytoflex (Beckman-Coulter). Se calcularon el acuerdo categórico, esencial y la desviación (*bias*) entre ambos métodos según la norma ISO/DIS10776-2:2021.

Resultados: La Tabla 1 describe los aislados incluidos en el estudio. La correlación obtenida entre los dos métodos estudiados se muestra en la Tabla 2. El acuerdo categórico fue del 100%, el esencial fue de 98.2% y el *bias* del +21.93%.

Conclusiones: Describimos un nuevo método rápido y preciso de determinación de la sensibilidad a colistina directamente de orinas positivas capaz de proporcionar resultados en 2h *versus* las 48h convencionales, que pensamos podera impactar en los laboratorios de microbiología clínica.

Enterobacterales	Especie	Pacientes	Inoculadas	Total
	<i>Escherichia coli</i>	29	9	38
	<i>Klebsiella pneumoniae</i>	7	17	24
	<i>Klebsiella aerogenes</i>	2	1	3
	<i>Serratia marcescens</i>		1	1
	<i>Proteus mirabilis</i>	1	3	4
	<i>Providencia rettgeri</i>		1	1
	Total			72
No fermentadores	Especie	Pacientes	Inoculadas	Total
	<i>Pseudomonas aeruginosa</i>		30	30
	<i>Acinetobacter baumannii</i>		12	12
	Total			42
Total estudio		49	65	114

Tabla 1: Distribución de especies y muestras utilizadas en el estudio

Método FASTinov	mg/L	Método de referencia (microdilución)									
		0,125	0,25	0,5	1	2	4	8	16	≥32	
	0,125		1								
	0,25	1	2	4							
	0,5		7	11	9						
	1		1	20	10	6					
	2			1	14	18	1				
	4					2					
	8							1			
	16										
	≥32									5	

Tabla 2: Correlación entre la CMI a colistina mediante el método de referencia y la técnica FASTinov®. Las CMIs dentro del acuerdo esencial se señalan en gris y los valores que fueron idénticos en ambos test están en gris oscuro.

- “A rapid colistin MIC determination directly from colonies, positive blood cultures or urines”; 27º Simpósio de Infecção e Sepsis, June 6 to 8, 2022; Ipanema Park Hotel Porto - **Poster**.



CERTIFICA-SE QUE:

Cidália Pina-Vaz

PARTICIPOU NO 27º SIMPÓSIO DE INFECÇÃO E SEPSIS, QUE SE REALIZOU NO IPANEMA PARK - PORTO, NOS DIAS 6, 7 E 8 DE JUNHO DE 2022.

E apresentou o Poster:

A RAPID COLISTIN MIC DETERMINATION DIRECTLY FROM COLONIES, POSITIVE BLOOD CULTURES OR URINES

Autores: Daniela Silva; Rosário Gomes; Inês Oliveira; Ana Dias; Ag Rodrigues; Rafael Cantón; Cidália Pina-Vaz



João Gonçalves Pereira
João Gonçalves Pereira



A rapid colistin MIC determination directly from colonies, positive blood cultures or urines

Daniela Silva^{1,2}, Rosário Gomes³, Blanca Pérez-Viso⁴, Inês Oliveira⁵, Ana Silva-Dias^{1,6}, AG Rodrigues^{6,7}, Rafael Cantón^{4,5}, Cidália Pina-Vaz^{1,6,7}

1. Instituto Português de Oncologia Porto Francisco Gentil, IPOPG, EPE, Serviço de Patologia Clínica, Porto; 2. Instituto de Ciências Biomédicas Abel Salazar, Mestrado em Oncologia Médica, Unidade Curricular Patologia Oncológica; 3. FASTinov, S.A., Porto, Portugal; 4. Servicio de Microbiología, Hospital Universitario Ramón y Cajal, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS) - Madrid (Spain); 5. CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, España; 6. CENTESIS - Center for Health Technology and Services Research, Faculty of Medicine, University of Porto, Porto, Portugal; 7. Division of Microbiology, Department of Pathology, Faculty of Medicine, University of Porto, Porto, Portugal;

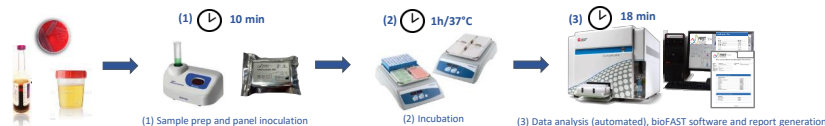


INTRODUCTION

Colistin is one of the “last-line” therapies to treat infections caused by multidrug-resistant Gram-negative bacteria when no other antibiotic options are available. Antimicrobial susceptibility testing (AST) is crucial for the selection of therapy, but colistin presents several technical issues on the available methodologies. Broth microdilution is the only AST method for colistin recommended by EUCAST/CLSI. It requires at least 18-24h to deliver results (growth-dependent method) from pure colonies, or almost 48 hours from positive blood cultures (BC) or urine. We here describe the performance of the FASTcolistin MIC developed by FASTinov® Porto (Portugal) already CE-IVD marked. It is a rapid AST assay based on flow cytometry for MIC colistin determination which delivers results in 1.5 hours when performed from either isolated colonies, positive BC or urines.

MATERIAL AND METHODS

A total of 427 samples were studied (253 colonies, 60 BC and 114 urines) using different species of *Enterobacterales*, *Pseudomonas* spp. and *Acinetobacter* spp. FASTcolistin MIC kit includes a panel with colistin in different concentrations (0.125-64 mg/L) combined with a fluorescent dye that shows cell damage, and bioFAST software which interprets and provides the MIC colistin value (interpreted according EUCAST/CLSI breakpoints). Results were compared with broth microdilution as reference method. Categorical (CA), essential agreement (EA) and bias were calculated, and interpretation was performed according to ISO 20776-2:2021. Reproducibility was also calculated. After a simple and swift extraction protocol, BC and urines were prepared to inoculate FASTcolistin MIC panel (urines that screened positive by Sysmex i1000 with 10⁵ CFU/mL in Gram-negative bacilli were included). A 0.5 McFarland bacterial suspension was prepared to inoculate FASTinov® panel directly from isolated colonies. Panels were incubated 1h at 37°C and analyzed by flow cytometry (Cytoflex, Beckman Coulter).



RESULTS

Overall results are detailed in Table 1. EA was > 90% and BIAS ≤ ±30% in line with ISO 20776-2:2021 criteria. CA was 99.5% according to EUCAST and 98.6% according to CLSI breakpoints. Reproducibility was 97% for both EUCAST and CLSI protocols.

Table 1: Overall results of EA and BIAS regarding each type of sample.

	n	EA	BIAS
Blood cultures	60	91.7%	+ 16.7%
Colonies	253	96%	+ 13%
Urine	114	93.9%	+ 22.8%
Overall	427	94.8%	+ 16.1%

CONCLUSIONS

We hereby describe a new accurate and rapid method ready to commercialization, for the determination of colistin MIC from colonies, or positive BC and urine samples that may have an impact on clinical microbiology laboratories and patient care due to the short time-to-result 1.5 h compared with the 24-48 h of standard methods.

- “Rapid colistin MIC determination by flow-cytometry directly from positive blood cultures”, **Daniela Fonseca e Silva, Sara Cruz, Rosário Gomes, Inês Martins-Oliveira, Ana Silva Dias, Blanca Pérez-Viso, Maria Helena Ramos, António Sarmento, Acácio G. Rodrigues, Rafael Cantón, Cidália Pina-Vaz**; 33rd ECCMID, April, 15 to 18, 2023, Copenhagen, Denmark – **Oral Communication**.



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Basel, 28th April 2023

ECCMID 2023 Oral presentation certificate

To whom it may concern:

We hereby confirm that the below abstract was submitted, accepted, and presented at the 33rd ECCMID [European Congress of Clinical Microbiology and Infectious Diseases], which took place in Copenhagen, Denmark, & online from 15 – 18 April 2023.

Title: Rapid colistin MIC determination by flow-cytometry directly from positive blood cultures

Abstract/Presentation Number: 2417/00786

Session Title: Last line antibiotics against pan-drug resistant Gram-negative bacteria

Session Type: 2-Hour Oral Session

Presenting author: D. Fonseca E Silva

co-Authors and Affiliation: D. Fonseca E Silva 1, S. Cruz 2, R. Gomes 3, I. Oliveira-Martins 3, 2, A. Silva-Dias 3, 2, 4, B. Perez-Viso 5, 6, M.H. Ramos 1, A. Sarmento 7, A.G. Rodrigues 2, 3, 4, R. Cantón 5, 6, C. Pina-Vaz 2, 3, 4.

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Sincerely yours,

Jacob Moran-Gilad
ECCMID Programme Director



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ABSTRACT

Key words: Colistin MIC, rapid AST, blood cultures, Flow cytometry AST

Objective. Colistin is a last line drug for treatment of multidrug resistant gram-negative bacilli in several countries. Its use varies with region and MIC determination is problematic given accuracy issues on most automated systems. Microdilution is the recommended method but cumbersome, taking 2 days to provide results from a positive blood cultures (BC) with standard protocols. The flow cytometry FASTcolistin MIC

developed by FASTinov® showed excellent accuracy when performed from pure colonies. In here we evaluated its performance directly from positive BC.

Methods. A total of 71 BC was assessed: 66 from patient's positive blood cultures from University-Hospital Centro Hospitalar S. João, Porto, Portugal (Feb-May 2022) sequentially obtained (only one from the same patient). Additionally, 5 spiked BC with ATCC strains [*E. coli* ATCC 13846 (*mcr1* positive), *E. coli* 25922, *K. pneumoniae* 13443, *K. pneumoniae* 700603 and *E. coli* 8739] were also included. Briefly, sample preparation was performed according Instructions for use taking max 5 min to obtain a clean bacterial suspension. The FASTcolistin MIC panel containing concentrations of colistin from 0.25 mg/L to 64 mg/L was then inoculated and incubated for 1-h followed by flow cytometry analysis (DxFlex, Beckman Coulter, USA). MIC was reported using a proprietary software and compared with the standard MIC determined by microdilution. Essential agreement (EA) and bias was calculated (ISO20776-2). Reproducibility was performed with 10 strains.

Results. Patient's blood cultures included 29 *E. coli*, 21 *Klebsiella* spp, 3 *Proteus mirabilis*, 3 *Serratia* spp, 1 *Morganella morganii*, 1 *Pantoea agglomerans*, 1 *Enterobacter asburiae* and 7 *Pseudomonas aeruginosa*. The MIC values obtained by flow-cytometry and standard methods for each sample is represented on fig.1. EA was 100% and BIAS +12.6%. Reproducibility was 98.1%.

Conclusion. FASTcolistin MIC kit from FASTinov® presents excellent accuracy, not only when performed from colonies but also directly from BC cultures with results in 2-h instead of 2 days.

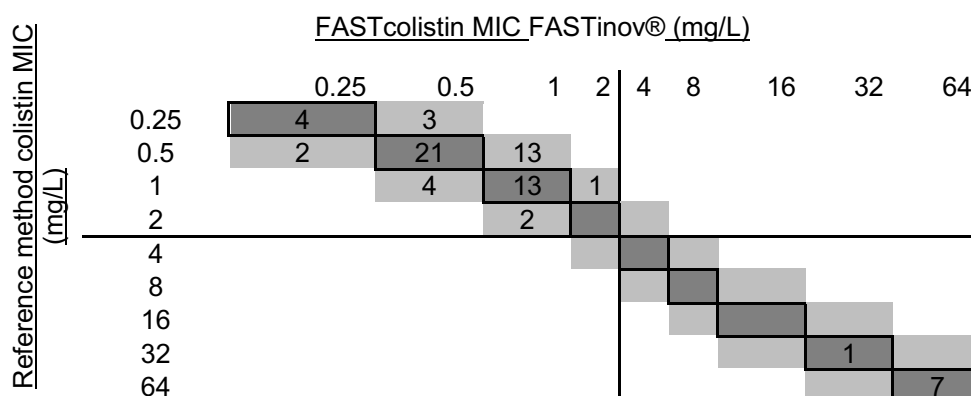


Fig. 1 – Distribution of the minimum inhibitory concentration (MIC mg/L) obtained with reference microdilution and flow cytometry (FASTcolistin MIC FASTinov®).

- **“IPO-Porto, o 1º hospital do mundo com antibiograma em 2h na resposta à sepsis”** Daniela Fonseca e Silva, Paulo Silva, Carla Campos, Iracema Romero, Gabriela Martins; IPO Porto Summit, May 9 and 11, 2024, in IPO-Porto – **Poster**.



ABSTRACT

Introdução: A sépsis é reconhecida pela OMS como uma situação clínica crítica que exige um diagnóstico mais rápido.

O IPO-Porto, com doentes críticos e imunodeprimidos, tem a necessidade na sépsis de diminuição de tempos de resposta.

Foi por isso o primeiro hospital a implementar o teste de suscetibilidade aos antibióticos(TSA) rápido directamente de hemoculturas(HC), desde fevereiro de 2024.

Objetivos: Dar a conhecer a metodologia a toda a comunidade hospitalar.

Descrição ou Materiais e Métodos: Segundo as metodologias clássicas (Walk-way(Biomerieux®)usado na rotina laboratorial do IPO-Porto) o TSA na sépsis era feito em 1-2 dias após a colheita de HC e sua positividade.

O TSA desenvolvido pela FASTinov (com painéis para bactérias Gram+ e Gram-) tem na sua metodologia a lise dos eritrócitos, a separação das células em centrífuga obtendo um pellet para identificação(ID) e posterior exposição por 1h, em estufa com

agitação, a diferentes antibióticos e fluorocromos. Este passo permite que na análise por citometria se observe se houve ou não lesão celular e correlaciona-la com a suscetibilidade(S) ou resistência(R). Ao final de 2h de positividade das HCs o TSA é disponibilizado pelo software da bioFast em amostras monobacterianas.

Resultados e Discussão: O resultado da ID diretamente a partir da HC usando o kit da FASTinov executada no MALDI-TOF (Brucker®), 30 minutos após positividade da HC, teve uma concordância de 100%(n=30) ao efetuado a partir das colónias (executado ao fim de 5h de positividade da HC após crescimento cultural).

Comparando o tempo de resposta do TSA, o tempo médio da FASTinov foi de 2h15min com um mín de 1h20min e um máx de 2h30min (casos em que foi processada mais do que uma HC ao mesmo tempo). Sendo consideravelmente mais curto do que o standard que teve um mínimo de 24h, um máx de 72h(fim-de-semana) sendo a media 45h.

Apresentamos casos clínicos nos quais o resultado foi antecipado 12 a 18 horas comparativamente com a rotina e na qual foi alterada a terapêutica.

Percebemos que essa alteração é mais evidente quando há interligação com o Serviço de Infecções.

Conclusão: Um resultado rápido do TSA permitirá, perante uma bactéria muito R aumentar o espectro da terapêutica e perante uma bactéria muito S reduzir este espectro com segurança em tempo útil e com valor clínico.

Deste modo este teste terá impacto significativo na optimização do tratamento dos doentes com sépsis.

Encontra-se em desenho um estudo de evidência clínica e impacto económico.

GUEST SPEAKER CERTIFICATES

- “Colistin activity evaluation through flow cytometry”; XVII online Congress of the Iberian Society of Cytometry, June, 15, 2021.



EACCME
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SPEAKER CERTIFICATE

This certificate declares that

Daniela Fonseca e Silva

participated as **Speaker** in the Session
“Microbiology: Clinical and flow cytometry application” with the lecture
“COLISTIN ACTIVITY EVALUATION THROUGH FLOW CYTOMETRY”
in the **XVII CONGRESS OF THE IBERIAN SOCIETY OF CYTOMETRY - Virtual Edition**
Porto, Portugal, from **14th to 18th June 2021**
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ORGANIZATION



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President of Iberian Society for Cytometry

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Chair Organizing Committee

- “Experiência Fastinov num hospital oncológico”; Quilaban Academy FASTinov – Serviço de Inovação para transformar o futuro do teste de susceptibilidade, November 22, 2023; Porto.



- “TSA por citometria de fluxo: vantagens e desvantagens”, 1ª Jornadas do Serviço de Patologia Clínica; December 18, 2023, IPO.Porto



PROGRAMA

10:00 • 10:30 **O papel da Citometria de Fluxo na LA Pediátrica** Bruno Fernandes (IPO-Porto)
 10:30 • 11:00 **Rastreio do HPV: Novas Guidelines** João Barreto (IPO-Porto)
 11:00 • 11:30 **Intervalo para café**
 11:30 • 12:00 **TSA por citometria de fluxo: Vantagens e desvantagens** Daniela Silva (IPO-Porto)
 12:00 • 13:30 **Intervalo para almoço**
 13:30 • 14:00 **O futuro da Patologia Clínica: Novos paradigmas no laboratório** José Duarte (IPO-Porto)
 14:00 • 14:30 **Estudo Hematológico de Líquidos Biológicos** Maria Luís Guerra (IPO-Porto)
 14:30 • 15:00 **Hiponatremia: Real ou fictícia?** Lília Cardoso (IPO-Porto)

Patrocínio



- “Antibiograma Rápido”, webinar - Associação Portuguesa de Técnicos de Análises Clínicas e Saúde Pública (APTAC), October, 24, 2024.



A Associação Portuguesa de Técnicos de Análises Clínicas e Saúde Pública (APTAC), certifica que

Daniela Fonseca e Silva

Participou, como Oradora no webinar “Antibiograma Rápido” no dia 24 de outubro de 2024, com a duração efetiva de 1:30h (uma hora e trinta minutos).



Membro de



Hélia Carona
 Hélia Carona
 Presidente Direção APTAC

- “Sepsis - Resposta laboratorial rápida no combate às resistências antimicrobianas”, World Antimicrobial Resistance Awareness Week, November, 22, 2024, i3S Porto.

3s

18 & 22 NOVEMBRO 2024
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WAAW'24
World Antimicrobial Resistance Awareness Week

18 NOVEMBRO 2024
AUDITÓRIO CORINO DE ANDRADE

12:00-13:00
SATELLITE SEMINAR
FILIPA GROSSO
I4HB, UCIBIO,
FACULDADE DE FARMÁCIA DA U. PORTO

22 NOVEMBRO 2024
AUDITÓRIO MARIANO GAGO

8:45
ABERTURA DO SECRETARIADO

9:00
SESSÃO DE ABERTURA
CLÁUDIO SUNKEL,
DIRETOR DO I3S

9:15-10:15 | SESSÃO I
LUÍS MALHEIRO,
UNIDADE LOCAL DE SAÚDE
DE GAIA E ESPINHO
ANDRÉ AMARAL GOMES,
HOSPITAL CUF PORTO
PAULO ANDRADE,
UNIDADE LOCAL DE
SAÚDE DE SÃO JOÃO, PORTO

10:30-11:30 | SESSÃO II
ANA CLÁUDIA CARVALHO,
UNIDADE LOCAL DE SAÚDE DE
BRAGA
DANIELA FONSECA E SILVA,
IPO, PORTO
RUTE OLIVEIRA,
I3S DIAGNOSTICS

11:30 | SESSÃO DE ENCERRAMENTO
DIDIER CABANES,
DIRETOR DO I3S DIAGNOSTICS

REGISTO
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WAAW'24
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18-22 NOVEMBER 2024

- “O antibiograma ultra rápido – soluções FASTinov”, Quilaban Academy, 20 de Novembro 2024, Coimbra

Quilaban ACADEMY

CERTIFICADO DE PRESENÇA

Certifica-se que
DANIELA FONSECA E SILVA
participou como orador(a) no Quilaban Academy
O ANTIBIOGRAMA ULTRA RÁPIDO – Solução FASTINOV
realizado a **20 de novembro de 2024**, em **Coimbra**.

Artur Silva
Assinado de forma digital por Artur Silva
Dados: 2024

Artur Silva
Diretor de área de Diagnóstico | Administrador

Quilaban
uma forma de vida

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Daniela Fonseca e Silva; Ferdinando F. Andrade; Rosário Gomes; Ana Silva-Dias; Inês Martins-Oliveira; Blanca Pérez-Viso; Maria Helena Ramos; Acácio G. Rodrigues; Rafael Cantón; Cidália Pina-Vaz. Ultra-rapid flow cytometry assay for colistin MIC determination in Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *Clinical Microbiology Infection*, 2020Nov;26(11):1559.e1-1559.e4. DOI: 10.1016/j.cmi.2020.08.019

Daniela Fonseca e Silva; Rita Teixeira-Santos; Ana Dias; Sara Oliveira; Inês Oliveira; Maria Helena Ramos; Acácio Gonçalves Rodrigues; Cidália Pina-Vaz. A novel flow cytometric protocol for the evaluation of colistin activity. *Clin Chem Lab Med* 2018;56(11):eA175,P07. DOI: 10.1515/cclm-2018-0636

Daniela Fonseca-Silva*; Rosário Gomes*; Inês Martins-Oliveira; Ana Silva-Dias; Maria Helena Ramos; Cidália Pina-Vaz. *Rapid determination of colistin susceptibility by flow cytometry directly from positive urine samples – Preliminary results*. *Int. J. Mol. Sci.* 2025, 26(3), 883. DOI: 10.3390/ijms26030883

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rapid colistin susceptibility directly from positive blood cultures using flow cytometry assay. International Journal of Antimicrobial Agents (IJAA). 2019Dec;54(6):820-823. DOI: 10.1016/j.ijantimicag.2019.08.016

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centro hospitalar
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Exma. Sra. Dra. Daniela Freitas
Serviço de Microbiologia

ASSUNTO: Trabalho Académico – Doutoramento - “Avaliação da suscetibilidade à colistina e monitorização de fármacos por citometria de fluxo” - N/ REF.º 2017.106(095-DEFI /079-CES)

O Conselho de Administração do CHP autoriza a realização do estudo acima mencionado, na prossecução dos objetivos 1 e 2 do estudo a realizar no Serviço de Microbiologia desta Instituição e tendo como Investigador Principal a Dra. Daniela Freitas. Quanto ao objetivo 3 da proposta, este, ficará sujeito a submissão posterior.

O estudo foi previamente analisado pela Comissão de Ética para a Saúde, pelo Gabinete Coordenador de Investigação, pela Direção do Departamento de Ensino, Formação e Investigação do CHP, e pela Direção de Enfermagem, bem como pelo Presidente do Conselho de Administração, tendo obtido parecer favorável.

Cumprimentos,

CONSELHO DE ADMINISTRAÇÃO
10/01/07
Dr. PAULO BARBOSA Presidente
Dr.ª ELIA GOMES Vice Executiva
Prof. Doutor JOSÉ BARROS Dr. RUI PEDROSO
Diretor Clínico
Enf.ª EDUARDO ALVES Enfermeiro Chefe

* Em todas as eventuais comunicações posteriores sobre este estudo é indispensável indicar a nossa ref.º.

COMISSÃO DE ÉTICA PARA A SAÚDE

APRECIÇÃO E VOTAÇÃO DO PARECER

Deliberação	Data: 26-7-2017	Órgão: Reunião Plenária
Título: "Avaliação da suscetibilidade à colistina e monitorização de fármacos por citometria de fluxo"		Ref.º: 2017.106(095-DEFI/079-CES)
Protocolo/Versão: TA - DT	Promotor: o(a) próprio(a)	Investigador: Dra. Daniela Santos Fonseca e Silva Pinto de Feitas - Médica Interna em Formação Específica de Patologia Clínica, Serviço de Microbiologia, Departamento de Patologia do CHP, Aluna do Programa Doutoral em Ciências Médicas, ICBAS.

A Comissão de Ética para a Saúde – CES do CHP, ao abrigo do disposto no Decreto-Lei n.º 97/95, de 10 de Maio, em reunião realizada nesta data, apreciou a fundamentação do relator sobre o pedido de parecer para a realização de TA - DT acima referenciado:

Ouvido o Relator, o processo foi votado pelos Membros da CES presentes:

Presidente: Dr.ª Luisa Bernardo

Vice-Presidente: Dr.ª Paulina Aguiar

Dr.ª Fernanda Manuela, Enf.ª Paula Duarte, Prof.ª Doutora Carla Teixeira, Prof.ª Doutora Maria Manuel Araújo Jorge, Dr. Gonçalo Senhorães Senra.

Resultado da votação:

PARECER FAVORÁVEL

A deliberação foi aprovada por unanimidade.

Pelo que se submete à consideração superior.

Data 26-7-2017

A Presidente da CES


Dr.ª Luisa Bernardo

APRECIÇÃO E PARECER PARA A REALIZAÇÃO DE TRABALHO ACADÉMICO - DOUTORAMENTO

Título: "Avaliação da suscetibilidade à colistina e monitorização de fármacos por citometria de fluxo"		Ref.º: 2017.106(095-DEFI /079-CES)
		Investigador: Dra. Daniela Freitas Serviço de Microbiologia

DIREÇÃO DE ENFERMAGEM: ☒ NÃO SE APLICA ☐ PARECER FAVORÁVEL ☐ PARECER NÃO FAVORÁVEL Data: _____	PRESIDENTE DO CONSELHO DE ADMINISTRAÇÃO: ☒ PARECER FAVORÁVEL ☐ PARECER NÃO FAVORÁVEL Data: 08/08/2017 Dr. PAULO BARBOSA Presidente do Conselho de Administração do CHP
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Informação:

Foram apreciados favoravelmente os objetivos 1 e 2 da proposta do estudo. Para a prossecução do objetivo 3 é necessária nova submissão ao CHP.

Em conformidade. Pode ser autorizado

pl1 
Prof.ª Doutora Luísa Lobato
Diretora do DEFI
03.08.2017