Simple phenotypic tests to improve accuracy in screening chromosomal and plasmid-mediated colistin resistance in gram-negative bacilli

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ABSTRACT

CLSI and EUCAST recommends that only broth microdilution (BMD) should be used for routine colistin susceptibility testing, however, it could be difficult to perform in resource-poor settings. The purpose of this study was to evaluate the accuracy of an agar spot test (COL-AS) and a colistin drop test (COL-DT) as compared to BMD. COL-AS and COL-DT were challenged with a collection of 271 Gram-negative bacilli clinical isolates: 195 Enterobacterales (including 63 mcr-1 positive strains), 37 *Acinetobacter* spp. and 39 *Pseudomonas aeruginosa*. For COL-AS, 3.0µg/ml (final concentration) of colistin was added to a Mueller-Hinton agar plate and subsequently swabbed with a 0.5 McFarland suspension of tested strain within 1cm^2 spot. For COL-DT, 10µl of a 16µg/ml colistin solution was dripped on the surface of a Mueller-Hinton agar plate, previously inoculated with a lawn of tested strain (0.5 McFarland). Colistin solution was made either, by dissolving powder or by disk elution in CA-MHB. Overall, 141/271 (52%) isolates were categorized as colistin resistant by reference BMD. COL-AS yielded a categorical agreement (CA) of 95.5% compared to BMD, with 0.7% very major errors and 3.8% major errors. COL-DT yielded a CA of 96.2% compared to BMD, with 0.7% and 0% very major errors and 3.1% and 3.8% major errors, for colistin powder and disk elution solutions, respectively. Most major errors occurred for mcr-1 producing strains with MICs that fluctuated from 2 to 4 µg/ml according to the method used. In conclusion, we developed and validated methods suited to the systematic screening of resistance to colistin in gram negative bacilli.

Clinical relevance: colistin continues to be one of the last-line therapeutic options to treat carbapenemase-producing gram negative bacilli. The BMD reference methodology, recommended by current standards for evaluating colistin sensitivity, is difficult to implement in laboratories from low-resource countries. Recently CLSI

endorsed two MIC-based alternative methods for testing colistin in Enterobacterales and *P. aeruginosa*, a colistin broth disk elution (CBDE) and a colistin agar test (CAT).In this work, we propose two simple methodologies, related to CLSI methods, to screen for colistin resistance, with a performance equivalent to the reference method in detecting resistance to colistin, both of plasmid (mcr) and chromosomal nature. Furthermore, the methods validated here allowed a better identification of those producers of mcr producer with borderline MICs. These screening tests can be routinely performed in addition to the tests currently in use, showed long stability during storage and some of them do not require colistin powder as the source of antibiotic, an important limitation in low-resource countries.

BACKGROUND

Colistin (polymyxin E) and polymyxin B are old polycationic peptides that have regained popularity as last resort treatments to face the worldwide emergence of multidrug-resistant gram-negative bacteria (1). Even though acquired resistance to polymyxins was seen only occasionally in the past, this is becoming more common because increased clinical and non-clinical use, such a veterinary growth promotion. (2). The recent identification of *mcr* genes, coding for plasmid-mediated resistance mechanisms, suggests that resistance to polymyxins might not only be mediated by chromosomal mutations but also spread by horizontal transmission, which warranted immediate worldwide attention (3, 4). These genes have been found primarily in *Escherichia coli* from human, animal, food and environmental samples (5, 6).

In 2020, CLSI issued colistin breakpoints for *Enterobacterales, Pseudomonas aeruginosa and Acinetobacter baumannii* complex but only considering intermediate (≤ 2 µg/ml) and resistant categories (≥ 4 µg/ml), based on the limited clinical efficacy of

this drug (7). While the clinical breakpoints issued by EUCAST for the same group of bacteria included susceptible (≤ 2 µg/ml) and resistant (> 2 µg/ml) categories (8). Both, CLSI and EUCAST guidelines recommend routine colistin susceptibility testing by estimation of MIC, being the broth microdilution-BMD- (without the addition of surfactant) the reference standard method (7, 8). However, there are important limitations for MIC estimation by BMD in several facilities, especially in resource-poor settings, because is a labor-intensive method and the accessibility to colistin sulfate.

MIC determination by other methods, such as gradient strips or semi-automatic equipment, as Phoenix or Vitek, have been discouraged by EUCAST (http://www.eucast.org/ast_of_bacteria/warnings/#c13111, last accessed 2nd July, 2020) or questioned in their performance due to very major errors in recent publications, respectively (9-13). In addition, the disk diffusion method is not reliable for measurements of *in vitro* colistin resistance because large molecular weight antimicrobials, as polymyxins, diffuse slowly into agar, resulting in small differences in the size of inhibition zones between susceptible and non-susceptible isolates (12, 14).

To solve the dilemma of how to best perform colistin susceptibility testing, in 2020, CLSI endorsed two MIC-based alternative methods for testing colistin in Enterobacterales and *P. aeruginosa*, a colistin broth disk elution (CBDE) and a colistin agar test (CAT). Both methods require the evaluation of serial two-fold colistin dilutions (range 1-4 µg/ml) in parallel with an unsupplemented control (7).

The objective of this study was to develop user-friendly colistin testing methods to BMD for screening colistin resistance. Tests developed in this work included a Colistin Agar Spot (COL-AS), a simplified version of the CLSI CAT, based on the addition of a unique concentration of a colistin solution on an agar plate with a simplified inoculation

protocol. Additionally, we proposed a colistin drop test (COL-DT), based on the application of a single drop of a polymyxin solution on the agar surface.

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MATERIALS AND METHODS

Collection and characterization of strains

A panel of previously characterized and de-identified 271 gram-negative clinical isolates, collected from a variety of sources (August 2012 to August 2017), belonging to the repository collection of the NRL, were used in this study. Strains were stored at -70ºC. Panel isolates were previously identified using classical biochemical tests or matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (16). Species distribution is depicted in Table S1. About 44 (1 *C. freundii*, 5 *Enterobacter spp*, 1 *E. coli*, 3 *K. oxytoca* and 36 *K. pneumoniae*) and 22 (1 *C. freundii*, 3 *E. coli* and 18 *K. pneumoniae*) isolates were KPC or NDM producers, respectively, as determined by PCR (17). Isolates were screened for the presence of *mcr*-1 genes by PCR, as described (18). *mcr*-1 positive *E. coli* isolates were compared by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA, as described (19).

Colistin susceptibility tests

Because polymyxins are able to bind to plastic surfaces, for all the tests, antibiotic stock solutions and dilutions were prepared and stored in glass bottles or tubes to limit as much as possible the contact of colistin with plastic. When required, glass pipettes were also utilized. The same batches of cation-adjusted Mueller Hinton broth (CA-MHB), MH agar (MHA) and colistin solutions were used in all the screening test, unless otherwise expressly indicated. All tests were performed on the same working day, for which the same bacterial suspension was used to inoculate the tests. The screening

Journal of Clinical Microbiology

methods were carried-out from fresh (18 to 24 h of incubation) mono-microbial cultures of the strains under study, growth in either plates of blood agar, chocolate agar, CLDE medium, TSA medium or MHA, according to availability.

1) Reference colistin susceptibility tests (BMD, macro-dilution and agar dilution)

Duplicate MIC values to colistin were obtained by the recommended BMD method, according to CLSI/EUCAST (8, 20), using in-house untreated 96-well sterile polystyrene microplates (Nunc, Roskilde, Denmark). Colistin sulfate powder (Sigma St. Louis, Missouri, USA) was dissolved in CA-MHB (BD, Franklin Lakes, USA) (stock solution, 128 µg/ml) and $\log_2$ dilutions were subsequently made to achieve a MIC range of 0.5 to 64 µg/ml. Cation levels (23.7 mg/L calcium and 11.2 mg/L magnesium) were verified by dry phase measurement (VITROS 4600 System, Ortho Clinical Diagnostics) after supplementation. The strains were considered to have acquired resistance to colistin when the MIC was higher than > 2 µg/ml, according to EUCAST standards (8). For colistin-susceptible *mcr* producers, MICs were confirmed by additional reference dilution methods (an agar dilution technique and a macro-dilution tube assay, 20) and by the recently proposed CBDE screening method (7).

2) COL-DT, Colistin Drop Test

The test described by Jouy E. et al. for *E. coli* was modified in the present work to make it suitable for other bacterial species (21). Briefly, we first tested two-fold concentrations of colistin sulfate solutions ranging from 4 to 64 µg/ml (solutions were the same used for BMD) with a reduced panel of isolates to select the optimal concentration (Table S1). The 16 µg/ml colistin solution showed the best performance in the preliminary tests and was subsequently tested against the entire panel.

A single 10 µl drop of the 16 µg/ml colistin solution was deposited on a MHA plate (Difco) previously swabbed with a 0.5 McFarland inoculum of the strain. The dripped

plates were left for 15 minutes at room temperature (the drop must be completely absorbed before moving the plate), then inverted and incubated for 16 to 18 h at 35 °C. After incubation, the presence or absence of an inhibitory zone was noted after carefully examination with transmitted light. For the purpose of standardization halos were recorded. An isolate was categorized as colistin susceptible if any zone of inhibition was observed, regardless of the diameter (median zones for susceptible isolates are about 10 mm for *Acinetobacter* spp and Enterobacterales and 8 mm for *P. aeruginosa*). An isolate was call as colistin resistant when there was no halo around the drop or when colonies within the inhibition zone, indicative of resistant subpopulations, were observed.

Subsequently, we tested additional two polymyxin solutions obtained by elution of commercial disks, namely: a 16 µg/ml colistin solution which was prepared by elution of eight 10 µg colistin disks (BD) in 5 ml volume of CA-MHB (BD). The same procedure was performed with eight 300 UI polymyxin B disks (Oxoid) in 10 ml of CA.MHB to obtain a 30 µg/ml polymyxin solution. Tubes containing disks were incubated at room temperature for at least 30 minutes but not longer than 60 minutes to allow polymyxins to elute from the disks, after which disks were aseptically removed. Solutions were sterilized by 0.22 µm filtration (Millipore) before use and kept un-fractioned at 4ºC. All solutions were dripped in the same agar plate, with each drop at least 2 cm away from the others. The stability of solutions was tested monthly for 12 months. Repeatability studies (triplicates, on consecutive days) were performed for selected isolates.

2) COL-AS, Colistin Agar Spot

In the spot test, the antimicrobial agent was incorporated into the (molten) agar medium

(MHA plates) as recommended for the agar dilution method (20), however, unlike agar dilution, the antimicrobial dilution to be tested is limited to the concentration/s that encompass the interpretive breakpoints. Colistin solutions were added to two batches of molten MHA (BD) to render 2.0 µg/ml and 3.0 µg/ml colistin (final concentration), respectively, in a 20-ml volume plate (100 x 10 mm). These concentrations were chosen because they separate the wild-type populations with MIC <= 2 µg/ml from non-wild-type with MIC > 2.0 µg/ml. Plates were kept in sealed bags at 4ºC up to 2 months. The excess moisture was removed before use by plate incubation for 5-15 min a 35ºC. Subsequently, a sterile cotton swab was dipped in a 0.5 McFarland suspension of the test strain and the excess liquid removed. Then, a spot was made on the agar surface of approximately 20 mm in diameter. The same plate allows up to 12 strains to be tested simultaneously. The inoculated plates were left for 15 minutes at room temperature, then inverted and incubated for 16 to 18 h at 35 °C. Plates were examined carefully with transmitted light. A strain was considered colistin resistant if exhibited >1 colony growth and colistin susceptible when there was no-growth at all. The presence of a single colony was considered possible cross-contamination and the test was repeated. The stability of spot plates was tested weekly for 2 months.

Additionally, we evaluated a commercial spot agar (ColTest, Laboratorios Britania, Argentina) that uses another brand of MHA (Britania) and a proprietary concentration of colistin, using the same procedure as the one described for the homemade tests.

Data analysis

Categorical agreement (CA) represented the rate of isolates grouped in the same susceptibility category by COL-DT or COL-AS compared with BMD, as recommended by the International Organization for Standardization (ISO) standard 20776-2 and the Federal Drug Administration guidance on review criteria for assessment of

antimicrobial susceptibility devices (23, 24). Very major errors (VMEs) were isolates that were susceptible to colistin by screening tests and resistant by BMD (MIC > 2 µg/ml); major errors (MEs) were isolates categorized as resistant by screening tests but susceptible by BMD (MIC <= 2 µg/ml). As we did not use intermediate category in our definition, no minor errors were analyzed. VMEs were calculated using the number of resistant isolates as the denominator, and MEs, using the number of susceptible isolates as the denominator. Agreement and error rates were evaluated by applying the requirements suggested by FDA for susceptibility testing methods (23, 24): a method must exhibit CA of >89.9%, a MEs rate based on the number of susceptible strains tested of < 3%, a VMEs rate of < 2.86%, based on the number of resistant strains included.

The positive (PPV) and negative predictive value (NPV) were also calculated as the number of true resistant/ number of resistant isolates and the number of true susceptible/ number of susceptible isolates, respectively.

RESULTS

Colistin reference MICs.

Overall, 141/271 (52%) isolates were categorized as colistin resistant by reference BMD: 17/37 (46%) *Acinetobacter* spp., 13/39 (33%) *P. aeruginosa* and 111/195 (57%) *Enterobacterales* (Table S1). The distributions of colistin MICs determined by BMD are presented in Figure 1. About 63/271 isolates, mostly *E. coli*, were found to produce *mcr*-1. All 61 *E. coli mcr* producers isolates were non-clonal. By BMD, 4/63 (6%) *E. coli mcr* producers repeatedly (triplicates) showed a MIC value below the cut-off value for colistin (MIC 2.0 µg/ml). Interestingly, no other enterobacteria had a colistin MIC of 2.0 µg/ml. Using a macro-dilution, the agar dilution and the CDBE, these 4 *mcr*-

producing strains, showed MICs one dilution higher, falling within the resistant category (Table S1).

Screening tests.

1) COL-DT

Overall, 96.2% of COL-DT results using colistin powder were in CA compared to the reference MIC. Most of strains (131/141) with colistin MIC > 2.0 µg/ml grew without an inhibition zone around the drop and the remaining 10 isolates, showed colonies within the zones. (Fig. 2; Table 1S). Only 1 VME (0.7%) and 4 ME (3.7%) were observed (Table 1). Interestingly, ME were exclusively for E. *coli mcr*-1 producers with a susceptible BMD (MICs of 2.0 µg/ml). When evaluated individually for each bacterial group, 100% CA was observed for *Acinetobacter* spp, 97.9% for Enterobacterales (0 VME, 4 ME) and 97.4% for *P. aeruginosa* (1 VME, 0 ME) (Table 1).

With a colistin solution made by elution of commercial disks, CA was also 96,2% compared to BMD. We observed 6 ME (3.8%), 4 of them were once again *E. coli mcr* producers with MICs of 2.0 ug/ml (Table 1S). VME were not observed. When evaluated individually for each bacterial group, CA was identical to that observed when colistin powder was used, except for *P. aeruginosa*, which was lower with the elution method (94.9% vs 97.4%) (Table 1). PPV and NPV are shown in Table 1.

With a polymyxin B solution made by elution of commercial disks, we obtained the lowest CA for this technique (93.4%), due to the 6 VME (3,5%) 4 ME (3.1%). VME were observed among all the bacterial groups and ME were associated with *E. coli mcr* producers with MICs of 2.0 ug/ml.

The stored solutions showed identical performance to those freshly prepared. Zones around the drop remained unchanged over 12 months. Likewise, the repeatability

248 studies showed agreement values (+/- 1 mm) in > 95% of the repetitions, without
249 changes in the categorization of the strains.

250 **2) COL-AS**

251 MHA plates supplemented with 2.0 µg/ml of colistin, showed 88.5% of CA with BMD,
252 with 15 VME (11.5%). CA improved substance up to 95,4% when colistin
253 concentration was increased to 3.0 µg/ml, regardless the brand of MHA used (Difco and
254 Britania) (Table 1). Most colistin resistant bacteria showed a confluent growth, except
255 5/141 and 4/141 with commercial brand and 3.0 µg/ml in-house test, respectively (Table
256 1S). Most semi-confluent growth was linked to *Salmonella* sp. (Figure 3). Remarkably,
257 no VME was observed in the spot test. In contrast, 5 and 6 ME were detected with 3.0
258 µg/ml in-house test and the commercial brand, respectively, mainly *E. coli* mcr
259 producers susceptible to colistin (MIC 2.0 µg/ml). When evaluated individually for each
260 bacterial group, the 3.0 µg/ml COL-AS showed 100% CA for *P. aeruginosa*, 94,6% for
261 *Acinetobacter* spp. and 97.9% - 98.5% for *Enterobacterales*. PPV and NPV are shown
262 in Table 1.

263 Test repetitions performed in consecutive days, as well those performed with the stored
264 plates up to 2 months, showed identical performance to those freshly prepared, without
265 changes in the categorization of the evaluated strains nor affecting the type and amount
266 of growth observed during storage (Table S1).

267

268 **DISCUSSION**

269 Colistin is the last resort agent used in the treatment of serious infections caused by
270 multi-resistant *Enterobacterales, P. aeruginosa* or *Acinetobacter* spp (1). Therefore, it
271 is essential for laboratories to report accurate results with good CA, being more
272 important than for many other antimicrobial agents when it comes to patients with

severe infections. Choosing an AST method for polymyxin susceptibility is challenging owing to poor penetration of drug in the agar medium, polycationic drug molecule binding to plates and changing MIC breakpoints by CLSI/EUCAST, as has happened in the last edition of CLSI (7), where only intermediate and resistant categories were issued. A critical step in the validation processes of new or improved AST techniques, is the inclusion of representative strains, both susceptible and resistant, to avoid bias towards a positive or negative result. For this reason, the panel of strains included in this work was selected to guarantee half (whenever possible) of representative isolates with resistance to the agent to be tested. It is also critical in the validation process to use the same bacterial inoculum across methods, since instability of the colistin resistant trait has been documented during the conservation, sub-culturing or thawing process of the bacterial frozen stocks (10, 25).

In this work, we validated simple and low-cost methodologies, specially conceived for low- or middle-income countries, with limited access to reference BMD. Methods proposed here could be simpler to adopt than the recent ones proposed by CLSI, as CBDE and CAT (7). Furthermore, these recently CLSI alternative methods do not included recommendations for *Acinetobacter*, being BMD the only approved method for colistin testing in this pathogen. Methods proposed in this work do not intend to replace MIC techniques, they would serve as screening to rule-out colistin-resistant strains from future analyzes.

The first methodology we recommend is the COL-DT, in which a single drop of colistin solution is applied on an inoculated agar surface. This technique was first used to test defensins against *Brucella* isolates (26) and, years later adapted to assess colistin susceptibility among *E. coli* of animal origin, including those with the *mcr*-1 (21). Under tests conditions described here, COL-DT had an excellent performance for

298 *Acinetobacter,* and Enterobacterales species other than *E. coli*. Regarding this bacterial
299 species, we observed ME >3%, corresponding to *mcr* producers with a borderline MIC
300 results, being susceptible by BMD but resistant by other reference methodologies, such
301 as agar dilution or macro-dilution and CBDE. Thus, the COL-DT, unlike the BMD,
302 classified all the *mcr*-producing isolates as colistin-resistant. Remarkably, *E. coli* with
303 BMD MIC of 2.0 µg/ml corresponded exclusively to isolates confirmed as producing
304 *mcr*-1. Therefore, a colistin MIC value of 2.0 µg/ml by BMD should be a call for
305 additional testing, either phenotypic, or genotypic (PCR) to confirm the presence of *mcr*
306 genes, especially among *E. coli,* as has been suggested by other authors as well (22).
307 Those laboratories that do not have access to colistin powder, will be able to obtain the
308 solution from a commercial colistin disk by elution. Use of commercial filter-paper disk
309 as the source of the antibiotic (broth elution MIC) to overcome the cost and the limited
310 access to powders, has been an old and widespread practice for anaerobic bacteria (27),
311 and is now recommended for the CBDE test included in M-100 standards (7). In this
312 work, the colistin solution obtained by disk elution had a performance equivalent to that
313 of the powder. Subsequently, we evaluated the performance of the COL-DT using the
314 BD Phoenix™ AST broth (Cat. 246003), ready-to-use MHB, cation-adjusted broth,
315 with identical results as those presented in this work (not shown). Additional
316 advantages of the COL-DT to other elution methods, include: i) the solution is stable for
317 more than 1 year in the refrigerator. ii) the test can be carried out on the same plate on
318 which paper disks have been placed to carry-out the disk diffusion technique, saving
319 consumables; iii) it has the potential to alert of possible sub-populations (28).
320 The use of polymyxin for the drop test is not recommended because the VME rate for
321 polymyxin elution was >3%. Usually, polymyxin B had demonstrated good correlation
322 with colistin in agar dilution and BMD tests (7, 29). However, this anticipated

correlation between both polypeptide antibiotics was not observed in the drop format, probably due to differential binding to the micropipette tips and/or a distinctive diffusion gradient around the dripped drop.

Numerous studies have demonstrated very good agreement between colistin agar dilution and BMD (30-34), with the exception of *P. aeruginosa* isolated from cystic fibrosis patients, in whom agar dilution might have more readily detected colistin resistance (30, 34). More recently, it was found that agar dilution was superior in terms of reproducibility and robustness, compared to broth dilution methods, for colistin MIC determination (34). Recently, an adaptation of the agar dilution method, named CAT, has been recommended by CLSI as a test for colistin resistance for *Enterobacterales* and *P. aeruginosa*: this method uses MHA plates supplemented with serial dilutions of colistin (1.0 µg/ml, 2.0 µg/ml and 4.0 µg/ml) that are subsequently inoculated with 10 µl of a 1/10 dilution of a suspension of the tested bacteria adjusted to the turbidity of 0.5 of Mc Farland. COL-AS is a modification of the recent CAT: we used a single colistin concentration MHA plate for screening for colistin resistance. We selected a concentration of 3.0 ug/ml which allowed to separate wild-type and non-wild-type subpopulation. Additional difference with the CLSI CAT included the inoculum type and inoculation procedure: we used direct inoculation of the 0.5 Mc Farland turbidity suspension, swabbing a defined area of the plate.

No VME were observed with the COL-AS, with a robust CA among *P. aeruginosa*, as observed for other agar-based methods (30, 34), and Enterobacterales other than *E. coli*. ME were mainly due to *E. coli mcr*-1 producers and *Acinetobacter* spp. On the contrary, CAT showed 3.9% VME (mostly among *Acinetobacter*) but no ME (22). The low number of *Acinetobacter* isolates in our study limits us for further comparisons between both methods.

An adaptation of the COL-AS proposed here was recently used for the specific detection of *mcr* producers, by adding an extra plate of colistin supplemented with EDTA, a well-known *mcr* inhibitor (35). Other agar-based colistin methods on the market were successfully utilized for screening purposes from clinical samples (36), showing the versatility of techniques based on agar dilution.

This study had several limitations: 1) we tested a limited number of *Acinetobacter* and *P. aeruginosa*. These observations may justify more extensive validation of these methods on this group of pathogens; 2) methods were evaluated with a limited number of brands of disks and/or culture media, therefore, for users of other brands should be considered provisional until additional data demonstrating adequacy is available; 3) this was a single-center study. Multicenter studies are required to further verify the results observed here.

In conclusion, we have developed and validated methods (COL-AS, ColTest and COL-DT), suited to the systematic screening of resistance to colistin in gram negative bacilli with a performance similar to the reference BMD. Due to the long stability, colistin solutions or plates could even be prepared centrally (i.e., in a reference lab) and distributed to peripheral laboratories in case they do not have the volumetric glass material requested for their preparation. These user-friendly screening tests can be routinely performed in addition to the tests currently in use.

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TRANSPARENCY DECLARATIONS

Nothing to declare

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Figure legends

Figure 1. Colistin MIC distributions obtained by BMD. Number of isolates with the indicated colistin MIC value according to bacterial group and the presence of *mcr*-1 gene. The dotted line indicates the breakpoint value that defines susceptible/resistant according to EUCAST standards (Ref. 8).

Figure 2. COL-DT. Examples of a colistin-resistant (a, c, d) and colistin-susceptible (b) strains by the drop test with 16 mg/L concentration of colistin in the 10 µL spot, obtained by powder solution or disk elution, as indicated. Strains a and c displyed well-defined colonies within the zone were categorized as resistant by the COL-DT. The species and the colistin broth microdilution MICs are indicated for each strain between brackets. The colistin gradient strip is shown only as indicative of the plane scale.

Figure 3. COL-AS. Examples of a colistin-resistant (b, c, d, e, f, g, h) and colistin-susceptible (a, i) strains by the in-house agar test with 3mg/L concentration of colistin. Strains c, d and f displayed semi-confluent growth. The species and the colistin broth microdilution MICs are indicated for each strain.

539 Figure 1.

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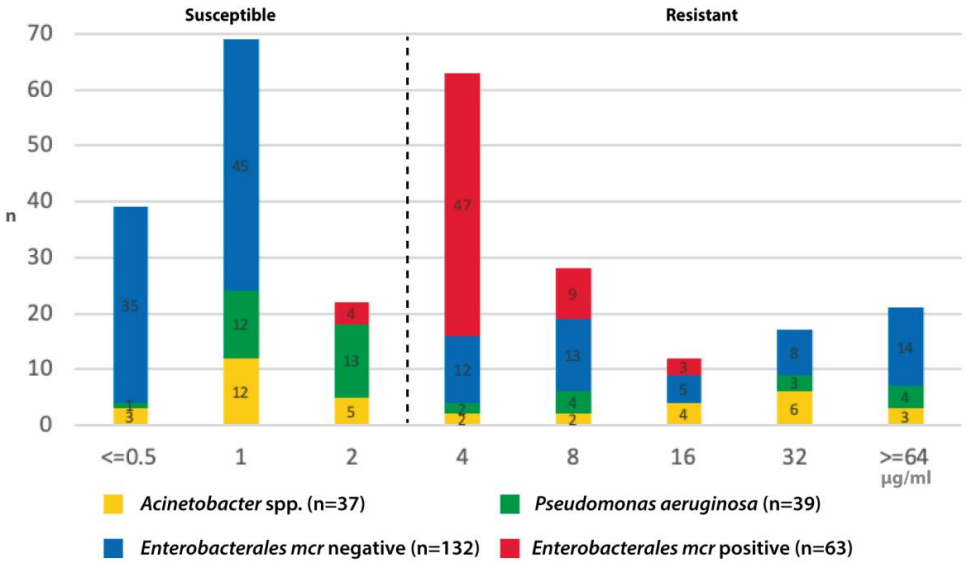
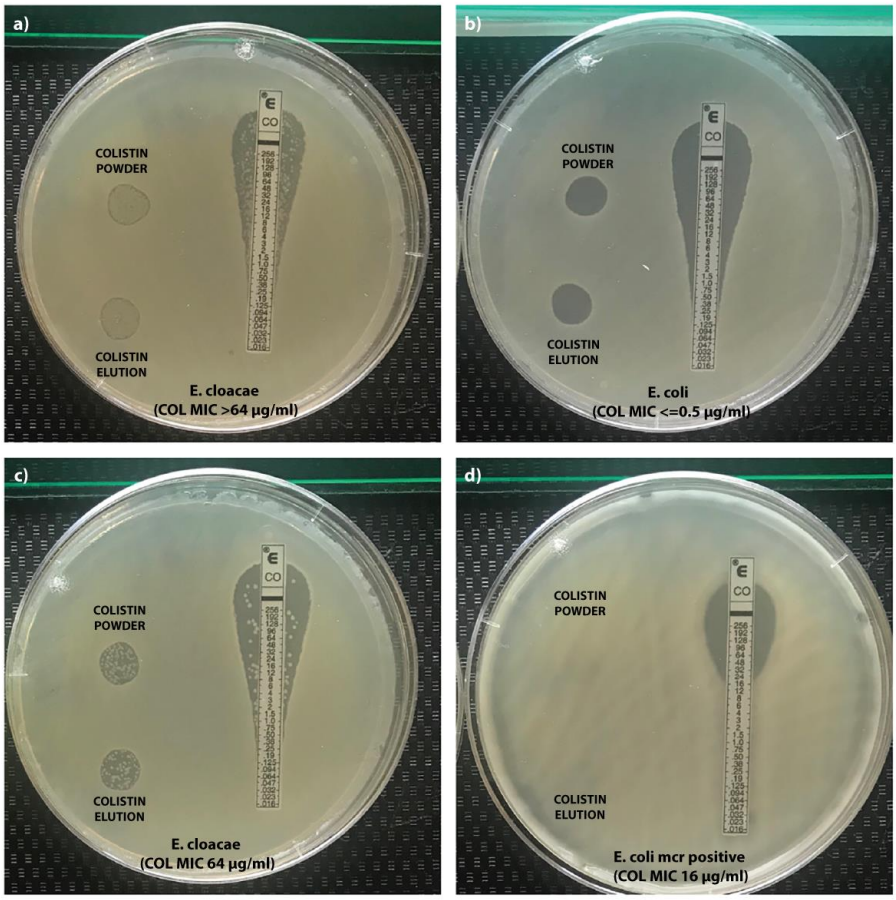


542 **Figure 2.**



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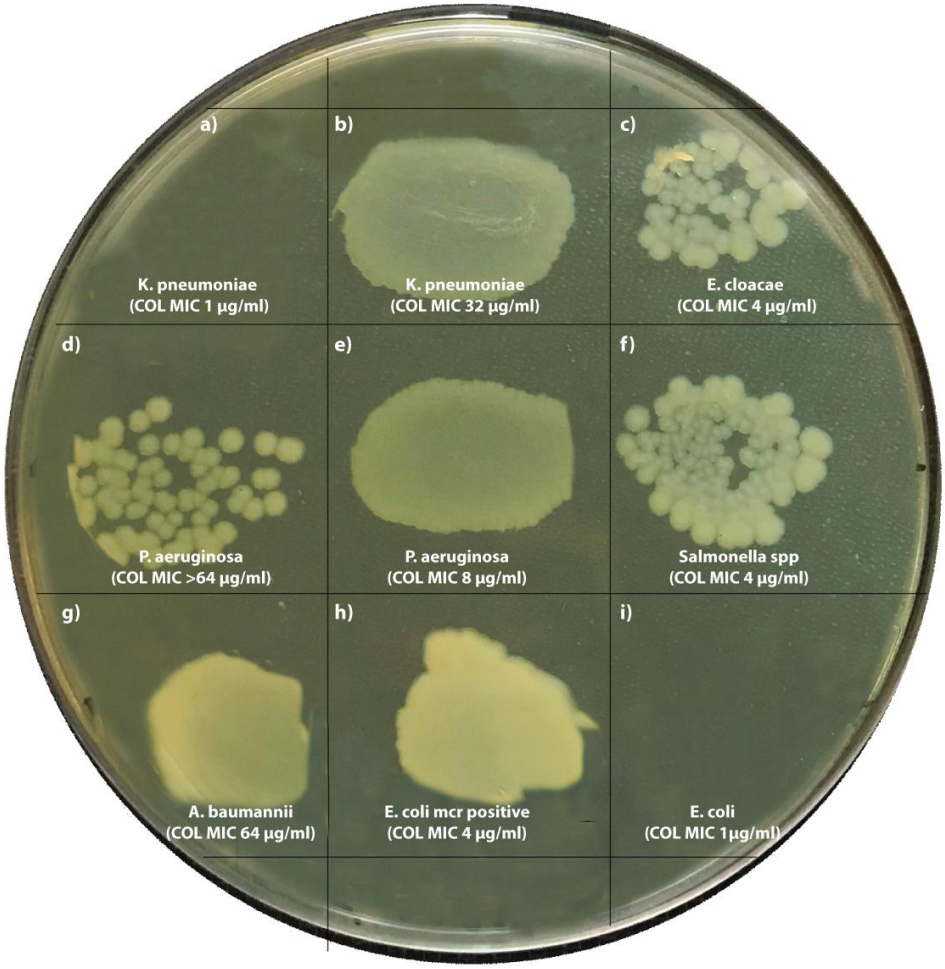
545 **Figure 3.**



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548 **Table 1. Summary of performance for COL-DT, POL-DT and COL-AS methods**

549 **compared to reference BMD MICs.**

Colistin screening tests	Group	Categorical agreement (%)	No. of (%)		Positive predictive value (%)	Negative predictive value (%)
			Very mayor errors	Mayor errors		
COL-DT (colistin powder)	**Overall**	**96.2**	**1 (0.7)**	**4 (3.1)**	**97.1**	**99.2**
	Enterobacterales (ETB)	97.9	0	4 (4.7)		
	Acinetobacter spp	100	0	0		
	P. aeruginosa	97.4	1 (7.7)	0		
COL-DT (colistin elution)	**Overall**	**96,2**	**0**	**6 (3.8)**	**95.7**	**100**
	Enterobacterales	97.9	0	4 (4.7)		
	Acinetobacter spp	100	0	0		
	P. aeruginosa	94.9	0	2 (7.7)		
POL-DT (polymyxin elution)	**Overall**	**93.4**	**6 (3.5)**	**4 (3.1)**	**97.1**	**95.4**
	Enterobacterales	96.4	3 (2.7)	4 (4.7)		
	Acinetobacter spp	97.2	1 (5.9)	0		
	P. aeruginosa	94.9	2 (15.4)	0		
COL-AS (2.0 µg/ml)	**Overall**	**88.5**	**0**	**15 (11.5)**	**92.2**	**100**
	Enterobacterales	95.9	0	7 (8.3)		
	Acinetobacter spp	89.1	0	4 (20)		
	P. aeruginosa	89.7	0	4 (15.4)		
COL-AS (3.0 µg/ml)	**Overall**	**95.5**	**0**	**5 (3.8)**	**96.4**	**100**
	Enterobacterales	98.5	0	3 (3.5)		
	Acinetobacter spp	94.6	0	2 (10)		
	P. aeruginosa	100	0	0		
ColTest (commercial agar spot)	**Overall**	**95.4**	**0**	**6 (4.6)**	**95.7**	**100**
	Enterobacterales	97.9	0	4 (4.7)		
	Acinetobacter spp	94.6	0	2 (10)		
	P. aeruginosa	100	0	0		

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