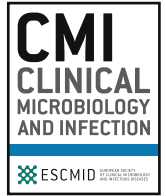




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## Original article

## Evaluation of ten brands of pre-poured Mueller-Hinton agar plates for EUCAST disc diffusion testing

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## ABSTRACT

**Objectives:** Mueller-Hinton agar (MHA) is recommended by EUCAST and CLSI for disc diffusion antimicrobial susceptibility testing (AST). We have previously investigated the quality of dehydrated MHA from several manufacturers. In this study, we evaluated the performance of ten commercial brands of pre-poured MHA plates.

**Methods:** AST was performed according to EUCAST methodology and results analyzed against targets and ranges in EUCAST quality control (QC) tables. MHA plates from different brands were tested in triplicate against four non-fastidious QC strains. The agar depth and pH were measured for all products. **Results:** The best performance was observed for MHA from Becton Dickinson (BBL MHA II), bioMérieux (MHE agar) and Hardy Diagnostics, for which >97% of zone diameters were within QC ranges and >60% on target  $\pm 1$  mm. The poorest performance was seen for plates from HiMedia (MHA and MHA no. 2), where 20% and 18% of readings were outside the QC ranges, respectively. The differences in pH and agar depth of the products were small and mostly within EUCAST specifications.

**Discussion:** The accuracy and reproducibility of disc diffusion AST depends on standardised procedures and high-quality discs and media. The performance among ten brands of pre-poured MHA plates differed significantly. The results indicate a poorer performance for pre-poured commercial plates as compared to in-house prepared plates from dehydrated powder of corresponding brands in our previous study. Manufacturers and clinical laboratories have a shared responsibility for the quality of AST. EUCAST provides QC criteria to be used both by manufacturers and laboratories. **Jenny Åhman, Clin Microbiol Infect 2022;•:1**

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## Introduction

Mueller-Hinton agar (MHA) was first described as the medium for standardised disc diffusion antimicrobial susceptibility testing (AST) of rapidly growing bacteria by Bauer et al. [1]. Today, MHA is recommended for disc diffusion by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [2] and the Clinical & Laboratory Standards Institute (CLSI) [3]. Disc diffusion is the most flexible, inexpensive, and rapidly adaptable of all AST methods. It is widely used for clinical AST, to screen for resistance mechanisms, and for antimicrobial resistance surveillance [4,5].

The components and requirements of MHA are specified in several published standards—International Standards Organisation [6], WHO [7], Food and Drug Administration [8], and Deutsches Institut für Normung [9]. MHA provides good growth of most non-

fastidious aerobic bacteria, and when supplemented with blood and/or other additives according to EUCAST [2] and CLSI [3], also of many fastidious microorganisms. MHA can be prepared in-house from dehydrated powder or obtained commercially as pre-poured agar plates from several international manufacturers.

For disc diffusion, as for MIC broth microdilution, a variety of components need to be tightly controlled. The end result is affected by the quality of MHA and antimicrobial discs [10,11], in addition to variables such as inoculum density, incubation time, incubation temperature, agar depth, pH of the medium, cation content, etc. To achieve accurate and reproducible results, laboratories must adhere to the standardised methodology and ascertain the quality of antimicrobial discs and MHA. In our previous studies, we discovered varying quality among 21 brands of dehydrated Mueller-Hinton (MH) media for in-house production of plates [10] and among antimicrobial discs from nine manufacturers [11].

The purpose of this study was to evaluate the performance of commercially available pre-poured MHA plates using defined culture collection strains with EUCAST quality control (QC) criteria [12].

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**Table 1**  
Product details for ten brands of pre-poured MH agar plates

MH agar brand	Manufacturer	Product code	Lot number	Expiry date	Recommended storage temp (°C)
BD BBL MH II Agar	Becton Dickinson and Company, Sparks, MD, USA	254032	0259477	2020-12-07	2–8
Biolife MH Agar II	Biolife Italiana, Milan, Italy	541740	2ML259	2021-01-23	2–8
bioMérieux MHE Agar	bioMérieux, Marcy l'Étoile, France	413822	1008332770	2020-12-31	2–8
Bio-Rad MH Agar	Bio-Rad Laboratories, Marnes-la-Coquette, France	63824	64372104	2021-03-23	2–20
E&O Laboratories MH Agar	E&O Laboratories, Burnhouse, Scotland, UK	PP0963	04153288	2020-12-14	2–8
Hardy Diagnostics MH Agar	Hardy Diagnostics, Santa Maria, CA, USA	G45	470595	2020-12-29	2–8
HiMedia MH Agar	HiMedia Laboratories, Mumbai, India	MP173	MPD0837	2021-05	20–30
HiMedia MH Agar no. 2	HiMedia Laboratories, Mumbai, India	MP1084	MPD0838	2021-05	20–30
Liofilchem MH II Agar	Liofilchem, Roseto degli Abruzzi, Italy	10031	101320086	2021-04-11	10–25
Oxoid MH Agar	Thermo Scientific, Wesel, Germany	PO5007A	4280832	2021-02-22	2–12

MH, Mueller-Hinton.

**Table 2**  
Antimicrobial agents, disc contents, and quality control strains

Antimicrobial disc	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>E. faecalis</i>
	ATCC 25922	ATCC 27853	ATCC 29213	ATCC 29212
Ampicillin 2 µg				●
Ampicillin 10 µg	●			
Piperacillin-tazobactam 30–6 µg		●		
Cefotaxime 5 µg	●			
Cefoxitin 30 µg			●	
Ceftazidime 10 µg	●	●		
Imipenem 10 µg		●		●
Meropenem 10 µg	●	●		
Ciprofloxacin 5 µg	●	●		
Norfloxacin 10 µg			●	●
Gentamicin 10 µg	●	●	●	
Gentamicin 30 µg				●
Tobramycin 10 µg		●		
Erythromycin 15 µg			●	
Tetracycline 30 µg			●	
Tigecycline 15 µg	●		●	●
Linezolid 10 µg			●	●
Trimethoprim-sulfamethoxazole 1.25–23.75 µg	●		●	●

## Materials and methods

Ten brands of pre-poured MHA plates (90 mm circular) from nine manufacturers were identified and included in the study (one manufacturer, HiMedia (Mumbai, India), offered two brands of MHA). Table 1 lists brand names and details on product code, lot number, expiry date, and recommended storage temperature for each product. The agar plates were ordered in October 2020 and tested as soon as all brands had been delivered (November 2020). All plates were stored according to the manufacturers' instructions throughout the study period. On the day of testing, the agar depth was measured three times each on two plates of each brand. Also, the pH of the media was measured (three times on two plates each) at 25°C using a surface electrode.

AST was performed at the EUCAST Development Laboratory, strictly adhering to EUCAST disc diffusion methodology [2]. The testing procedure was identical to that used in our previous study on dehydrated MH powder [10] and included four non-fastidious QC strains recommended by EUCAST for routine QC and 18 antimicrobial discs representing different antimicrobial classes (Table 2). All discs were from one manufacturer (Oxoid; Thermo Fisher Scientific, Basingstoke, UK), selected because of their accurate performance in our previous disc study [11]. Each disc-strain combination was tested in triplicate (three separate inoculum suspensions), and each suspension applied to the ten different

MHA plates in parallel. All inhibition zone diameters were measured by a single technician to the nearest millimetre using a calliper. This resulted in a total number of 900 zone diameter readings (90 per agar).

Four MHA brands had a shelf life of >4 months—Bio-Rad (Marnes-la-Coquette, France), HiMedia (MHA and MHA no. 2), and Liofilchem (Roseto degli Abruzzi, Italy). To investigate performance over time, test procedures were repeated at 4, 8, 12, and 18 weeks following the first test occasion. The pH and agar depth were measured on each occasion. All tests were performed within the shelf life of the products.

## Data analysis

The disk diffusion results were analyzed against target values and ranges in the EUCAST QC Tables v11.0, 2021 [12]. The mean value of triplicate tests of each disc-strain combination was given a rating using the same procedure as in our previous study—0 points if on target  $\pm 1$  mm, minus 1 point if on target  $\pm 2$  mm (but not  $\pm 1$  mm), minus 3 points if > 2 mm from target but within the QC range, and minus 5 points if outside the QC range. The summary of the ratings given for each QC strain resulted in a total rating for each MHA. In theory, the highest total rating was zero (all mean values on target  $\pm 1$  mm) and the lowest rating -150 (all mean values outside range). For each MHA brand, the percentages of individual zone diameters being within target  $\pm 1$  mm and those outside range were also calculated. The pH and the agar depth were evaluated against EUCAST specifications [2].

## Ethical considerations

There were no patient strains involved in this study. All testing was performed *in vitro* using type culture collection strains.

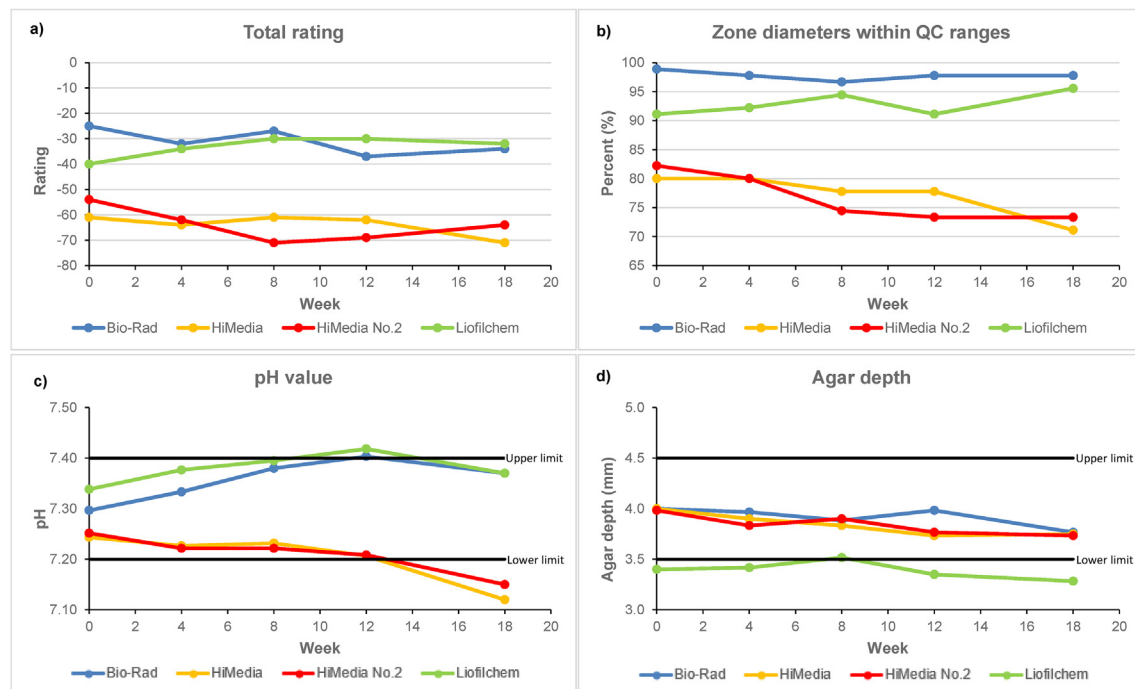
## Results

All tests resulted in confluent growth of the four QC strains for all MHA brands. Of the total 900 inhibition zone diameter readings, 93% ( $n = 840$ ) were within QC ranges and 52% ( $n = 466$ ) were on target  $\pm 1$  mm. The total rating for each brand ranged from -11 to -61 (Table 3). Table 3 shows the summarised results for all parameters analyzed. The best ratings were observed for pre-poured MHA from Becton Dickinson (BD; BBL MHA II; Sparks, MD, USA), bioMérieux (MHE agar; Marcy-l'Étoile, France), and Hardy Diagnostics (Santa Maria, CA, USA), for which >97% of zone diameter readings were within QC ranges and >60% were on target  $\pm 1$  mm. The poorest ratings were observed for MHA plates from HiMedia

**Table 3**  
Performance of ten brands of pre-poured Mueller-Hinton (MH) agar plates

Total rating <sup>a</sup>	MH agar brand	Product code	pH	Agar depth (mm)	Percent zones on QC target $\pm 1$ mm	Percent zones outside QC range	Agents outside range, high	Agents outside range, low
-11	BD BBL MH II Agar	254032	7.28	4.0	70	0.0		
-14	bioMérieux MHE Agar	413822	7.17	4.3	66	2.2	TS	
-17	Hardy Diagnostics MH Agar	G45	7.26	4.5	60	0.0		
-22	Oxoid MH Agar	PO5007A	7.20	4.0	56	3.3	TS	
-23	E&O Laboratories MH Agar	PP0963	7.22	4.0	58	5.6	CA	TE
-25	Bio-Rad MH Agar	63824	7.30	4.0	48	1.1	AM	
-30	Biolife MH Agar II	541740	7.24	4.0	50	7.8	AM, TS	
-40	Liofilchem MH II Agar	10031	7.34	3.4	50	8.9	AM, TS	
-54	HiMedia MH Agar no. 2	MP1084	7.25	4.0	34	18	PC, CS, CA, FQ, AM, TS	
-61	HiMedia MH Agar	MP173	7.24	4.0	27	20	PC, CS, CA, FQ, AM, TS	

AM, aminoglycosides; CA, carbapenems; CS, cephalosporins; FQ, fluoroquinolones; MA, macrolides; PC, penicillins; TE, tetracyclines; TS, trimethoprim-sulfamethoxazole.  
<sup>a</sup> Rating based on how mean values from triplicate tests of each quality control (QC) strain relate to the respective QC criteria (EUCAST QC Tables v11.0 [12]): 0 points if within target  $\pm 1$  mm, -1 point if within target  $\pm 2$  mm (but not  $\pm 1$  mm), -3 points if  $> 2$  mm from target but within QC range, and -5 points if outside the QC range.



**Fig. 1.** Performance over 18 weeks (5 test occasions) for four brands of pre-poured Mueller Hinton agar plates, showing a) total rating for all quality control (QC) strains tested, b) percentage of zone diameters within QC ranges, c) agar surface pH and d) agar depth.

(MHA and MHA no. 2), where 20% and 18% of readings were outside the QC ranges and only 27% and 34% on target  $\pm 1$  mm, respectively.

Results outside the QC ranges were observed for 6.7% (60/900) of all zone diameter readings. Of those, most results (58/60) were above and only two results were below the QC limits. The antimicrobial agents with most out-of-range results were trimethoprim-sulfamethoxazole (14/90), aminoglycosides (23/150), and carbapenems (9/120), especially meropenem. For results on individual agents and QC strains, see web-only Supplementary materials, Tables S1–S5.

#### Agar depth and pH

All measurements of the agar depth were within the defined limits 3.5 to 4.5 mm, except for Liofilchem MHA for which 4 of 6 measurements were below 3.5 mm. The pH values were within specified limits (7.2–7.4) for all brands, except for MHE agar from bioMérieux for which all measurements were below the limits and

the mean value was 7.17. The mean pH and agar depth for each product are presented in Table 3.

#### Performance over time

Four MHA brands with expiry dates permitting longitudinal analysis were tested five times during four months (Fig. 1(a–d)). The two HiMedia products (MHA and MHA no. 2) were clearly affected, during four months the total ratings and the proportion of zone diameters inside the QC ranges decreased even further. For these products, the pH decreased to values below specified limits and this was associated with out-of-range results, especially for gentamicin and imipenem with *Enterococcus faecalis* ATCC 29212. For Bio-Rad and Liofilchem products, the performance was stable during the four months, although the amount of agar in the Liofilchem plates was inappropriately low throughout. After four months the pH was slightly higher, but within limits, for MHA from Bio-Rad and Liofilchem.

## Discussion

The EUCAST Development Laboratory has previously evaluated the quality of antimicrobial discs from nine manufacturers [11] and the quality of 21 brands of dehydrated MH powder for in-house production of plates [10]. In this study, we investigated the performance of ten brands of commercially available pre-poured MHA plates for disc diffusion susceptibility testing. All plates were bought commercially without the manufacturers' knowledge of the upcoming evaluation and tested shortly after receipt (well within expiration dates). The products could not be tested blindly since the manufacturers are obliged to label the Petri dishes. Testing strictly followed the standardised disc diffusion methodology, and variations due to inoculum density, incubation time, and temperature were minimised by testing all brands in parallel from the same inoculum suspension. Variations in zone diameter reading were minimised by all readings being performed by the same technician.

The performance of the ten MHA brands differed significantly. A few brands had 100% of results inside QC ranges, whereas others had up to 20% of results outside QC ranges. The two products with the poorest ratings (HiMedia MHA and MHA no. 2) had out-of-range results for most antimicrobial classes tested. The use of such agar plates for AST of clinical isolates will cause incorrect susceptibility categorization. Since out-of-range results were almost exclusively above the QC ranges (zone diameters too large), false susceptible results can be expected. Both EUCAST and CLSI recommend MHA for disk diffusion AST, and whenever the QC strain and disc content is the same, the QC criteria are identical. Systematic deviations will therefore apply to both systems.

Poor results may be related to differences in thymidine and/or cation content of the media, as seen in our previous study on dehydrated media [10]. The concentrations of calcium and magnesium in the media are known to affect the activity of aminoglycosides. This can be controlled by testing *Pseudomonas aeruginosa* ATCC 27853 with an aminoglycoside [13]. For HiMedia MHA and MHA no. 2, all results for gentamicin and *P. aeruginosa* ATCC 27853 were above the QC ranges, indicating low concentrations of divalent cations. The most common agent with out-of-range results in this study, as well as in our previous study, was trimethoprim-sulfamethoxazole, for which the activity is strongly affected by the concentration of thymine and thymidine in the agar. In our previous study on dehydrated MH media, the content of several cations were analyzed, however, in this study where plates were delivered as ready-made products, we did not measure the levels of different components. The pH of the media will affect the activity of several agents, such as macrolides, aminoglycosides, and tetracyclines, and the agar depth is directly related to inhibition zone formation for all antimicrobial classes. In this study, the differences in pH and agar depth of the ten products were small and mostly within specified limits.

Nine of the ten brands in this study were also included in our study on dehydrated media for in-house production of plates [10]. In the two studies, we used the exact same procedures for evaluating the media. For seven of nine brands, the total ratings were in favour of in-house prepared plates as compared to pre-poured plates of the same basic media. One brand, BBL MHA II, had identical ratings for in-house produced and pre-poured plates. With plates from BD (BBL) and Hardy Diagnostics, all zone diameters were within the defined QC ranges.

The pre-poured MHA brands differed in shelf life, packaging, and recommended storage temperatures. One manufacturer (Hardy Diagnostics) stated that plates were packed in breathable cellulose bags to prevent condensation and excess moisture, and another manufacturer (HiMedia) included desiccants in the

plastic bags. HiMedia and Liofilchem recommended storage of MHA in room temperature, whereas, the other manufacturers recommended plates to be stored refrigerated. The shelf life of commercial plates is generally longer than that assigned to plates produced in-house. Brands with a long shelf life (>4 months) were investigated for performance over time. For MHA from Liofilchem and Bio-Rad we could not detect a change in performance during 4 months although the agar depth of plates from Liofilchem was inappropriate. For MHA and MHA no. 2 from HiMedia the performance was poor already from day one, and after 4 months even poorer.

The quality of commercial MHA plates is affected by the production procedure, the quality of the ingredients (agar, peptones, etc.), autoclaving, packaging, transportation, and storage. A previous study reported variation in quality between different lots of pre-poured MHA plates from four manufacturers [14]. This study points to differences in performance among ten brands of pre-poured MHA plates. Our results also indicate that pre-poured commercial plates do not perform as well as plates made in-house from dehydrated MH media [10]. Although fastidious organisms and MHA with supplements to support such organisms were not investigated in this study, experience shows that when the basic MH media does not meet QC criteria, the system will fail also for other organisms.

When EUCAST disc diffusion criteria are developed, discs and MH media from several manufacturers are included [15] aiming to create a robust system designed to tolerate some variation related to the materials used. The EUCAST QC criteria, including targets and ranges, aim to support manufacturers in their development of AST materials (discs, media, gradient tests, devices) and clinical laboratories in their routine susceptibility testing. For manufacturers, the accuracy of the product is determined by its ability to produce results close to the target value. The range in EUCAST QC tables has little or no value for manufacturers but helps clinical laboratories ascertain that day-to-day random variation of the entire AST procedure is within acceptable limits. The mean value of repeat testing of QC strains in the clinical laboratory should optimally be within  $\pm 1$  mm of the target value.

For commercially prepared MHA plates, the laboratory should follow the manufacturers' recommendation on storage and shelf life and check that the agar depth for each batch is 4 mm. The internal quality control system of the laboratory should be organised to discover deviations in media and disc performance, and accreditation authorities should make sure that this is included in quality control systems. QC performed by the manufacturer of prepared media cannot serve as the only quality control. Any batch which does not meet quality criteria should be disused and the manufacturer immediately notified. Using media of poor quality will affect the susceptibility test results and may lead to erroneous susceptibility categorisation. The final responsibility for AST results is with the clinical laboratory.

## Conclusions

Disc diffusion remains the most inexpensive, versatile, and widely used method for phenotypic susceptibility testing of bacteria. Apart from using a highly standardised procedure, the accuracy and reproducibility of the method will depend on the quality of discs and media employed. The EUCAST method has an inbuilt buffer for variation in that it was developed using discs and media from several manufacturers. This study evaluated the performance of ten brands of pre-poured MHA plates from international manufacturers. The performance differed significantly— some products met the QC criteria and exhibited the correct pH and agar depth, whereas other products had severe problems with out-of-range

results for many or even most antimicrobial classes. Results with pre-poured media were generally poorer than when the same dehydrated powders were used to produce plates in-house in our previous study [10]. However, many laboratories do not have the option to produce media in-house. Manufacturers of AST materials (discs, dehydrated powders and pre-poured plates) have a responsibility to meet the quality criteria and targets published by EUCAST, but the final responsibility for AST results lies with the individual laboratory. Each laboratory must confirm that materials and results meet published quality criteria.

### Transparency declaration

The authors declare that they have no conflict of interest. The study was not sponsored by any commercial company.

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### Author contributions

JÅ performed the antimicrobial susceptibility testing. JÅ, EM, and GK planned the study and analyzed and evaluated the results. All of the authors contributed to writing the manuscript.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cmi.2022.05.030>.

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