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EUCAST evaluation of 21 brands of Mueller–Hinton dehydrated media for disc diffusion testing

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ABSTRACT

Objectives: Mueller–Hinton (MH) agar is recommended by EUCAST and CLSI for disc diffusion antimicrobial susceptibility testing. Using EUCAST methodology, we evaluated the performance of 21 internationally available brands of dehydrated MH agar from 17 manufacturers.

Methods: MH plates were prepared in-house and evaluated against four quality control (QC) strains tested in triplicate, using EUCAST disc diffusion methodology. This resulted in 30 disc–QC strain combinations and 90 readings per MH brand. All brands were tested blindly and in parallel. Results were evaluated against targets and ranges in the EUCAST QC tables. The agar depth, pH and concentration of five cations were measured for all brands.

Results: Six brands of MH agar (Bio-Rad, Biolife, Oxoid, Sigma MH 2, BD BBL MH II and CRITERION) demonstrated excellent performance, with $\geq 99\%$ of zone diameter readings within QC ranges and $\geq 70\%$ on target ± 1 mm. The poorest performance was seen for Biolab and Merck MH, with 10% (9/90) and 23% (21/90) of readings outside the QC ranges, respectively. Of all readings, 4.9% (93/1890) were out of range, mainly related to trimethoprim sulfamethoxazole ($n = 25$), aminoglycosides ($n = 25$) and fluoroquinolones ($n = 15$). The cation content differed considerably between the agars, and for four brands pH values were outside the acceptable range 7.2–7.4.

Discussions: This study evaluated the performance and content of 21 brands of MH dehydrated media. Six brands showed excellent performance with all investigated antimicrobial classes. Others exhibited problems with one or more classes of agents. This could partly be explained by differences in concentration of specific chemical components and pH. **J. Åhman, Clin Microbiol Infect 2020;:1**

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Introduction

The purpose of *in vitro* antimicrobial susceptibility testing (AST) is to provide guidance for clinical therapy. With increasing resistance rates the use of standardized, simple, flexible and inexpensive methods, such as disc diffusion, has become increasingly important. Mueller–Hinton (MH) agar was originally developed in 1941 by John Howard Mueller and Jane Hinton [1]. In 1966, Bauer et al. described a standardized disc diffusion AST method based on MH agar [2]. Today, MH agar is used worldwide and recommended for disc diffusion by the Clinical & Laboratory Standards Institute (CLSI) [3] and The European Committee on Antimicrobial Susceptibility

Testing (EUCAST) [4]. MH agar is also used for agar dilution testing and the basic formula as MH broth for reference broth microdilution susceptibility testing [5].

MH media contains beef extract and casein hydrolysate to provide nitrogen, vitamins, carbon, amino acids and other nutrients to support the growth of microorganisms. Agar acts as the solidifying agent and starch is added to absorb any toxic substances in the medium. Unsupplemented MH provides satisfactory growth of most non-fastidious pathogens and when supplemented with mechanically defibrinated horse blood (5%) and β -nicotinamide adenine dinucleotide (20 mg/L) also many fastidious microorganisms [4]. MH agar can be obtained from many international manufacturers as commercially pre-poured agar plates and as dehydrated powder for in-house preparation of agar plates.

For manufacturers, there are several published standards describing the requirements for MH agar: ISO [6], WHO [7], FDA [8] and DIN [9] (Table 1). For manufacturers and users, criteria for

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Table 1
Requirements for Mueller–Hinton agar in four standards

Standard	DIN 58940-3: 2008-10	WHO 28th report, 1977	ISO/TS 16782: 2016	FDA BAM, 8th ed. Rev. A, 1998
Meat infusion (g/L)	2.0			
Dehydrated infusion from beef (g)		300	300 ^a	300
Casein hydrolysate (g/L)	17.5			17.5
Acid digest of casein (g/L)		17.5	17.5	
Starch (g/L)	1.5		1.5	1.5
Corn starch (g/L)		1.5		
Agar (g/L)	12–18	17	17	17
Ca ²⁺ (mg/L)		50–100		
Mg ²⁺ (mg/L)		20–35		
Mn ²⁺ (mg/L)			<8.0	
Zn ²⁺ (mg/L)			<3.0	
Thymidine (mg/L)			<0.03	
Agar depth (mm)	3.0–4.0	4.0	3.5–5.0	
pH at 25°C	7.2–7.4	7.2–7.4	7.2–7.4	7.3 ± 0.2

^a 2 g of beef extract powder.

controlling the performance of MH agar are available from EUCAST [10] and CLSI [11].

To obtain reliable and reproducible results, laboratories must follow the standardized methodology without modification and use materials of good quality. Although the quality of discs and media is mostly in the hands of the manufacturers, the final responsibility for the correctness of AST results rests with the laboratory. The properties of MH agar, such as pH [12], agar depth [13] and the content of divalent cations [14–17], and thymidine [18,19], will affect inhibition zone formation. In this study, we aimed to identify all manufacturers of dehydrated MH agar and evaluate the performance against defined type culture collection strains (QC strains) with EUCAST quality control (QC) criteria, in the form of defined target values and acceptable ranges.

Methods

Eighteen antimicrobial discs (Table 2) were used to test 21 brands of MH dehydrated agar powders from 17 manufacturers (Table 3). Four manufacturers produced two different brands of MH agar each. Table S1 lists brand names and details on product code, lot number and expiry dates of each product. MH plates were prepared in-house from 21 dehydrated powders (one lot per brand) on one day according to the instructions of the respective manufacturer. The agars were autoclaved at 121 °C for 15 min and poured (26 mL per plate) using an automated dispenser (Integra MediaJet, Integra Biosciences, Zizers, Switzerland) into 90-mm circular Petri dishes (Sarstedt, Nümbrecht, Germany) to a uniform agar depth of 4 ± 0.5 mm [20]. The pH of the solidified agar was measured (15 values per agar brand) at 25 °C using a surface electrode. The agar depth was measured (six values per brand) as the thickness of punched pieces of agar using a calliper. To ensure that all testing was unbiased plates from different brands were randomly numbered 1–21 by otherwise uninvolved technical staff. The products were decoded once all testing and data analysis were finalized.

AST was performed at the EUCAST Development Laboratory (EDL) using EUCAST disc diffusion methodology [4]. Testing included four non-fastidious QC strains recommended by EUCAST and 18 antimicrobial discs (one lot of each), chosen to represent different agent classes and to include agents that could reveal effects of varying pH and contents of cations and thymidine (Table 2). Discs from Oxoid (Thermo Fisher Scientific) were used due to their accurate and reproducible performance in a previous disc evaluation study [21]. Each disc strain combination was tested in triplicate on the same day, using three individually prepared inoculum

suspensions. Each suspension was applied to the 21 agar brands in parallel, to minimize variations due to differences in inoculum and incubation. Inhibition zone diameters were measured by a single technician to the nearest millimetre using a calliper. This resulted in a total number of 90 readings (30 disc–QC strain combinations tested in triplicate) per MH brand (Table 2).

Analysis of cations in agar

The concentrations of five ions were measured in each dehydrated powder: calcium (Ca), magnesium (Mg), zinc (Zn), manganese (Mn) and iron (Fe). The analysis was performed at SYNLAB Analytics & Services Sweden AB (Linköping, Sweden) using inductively coupled plasma-atomic emission spectroscopy, ICP-AES (iCAP 7000 Series, Thermo Scientific, UK), according to ISO 11885: 2007 [22]. Prior to analysis, the agar powders were decomposed by digestion in aqua regia (75% hydrochloric acid, 25% nitric acid), refluxed for 2 hr at 120 °C and after cooling diluted in deionized water to clear solutions. The measured concentrations (mg/kg) were recalculated to final concentrations in agar (mg/L) as prepared according to each manufacturers' instructions.

Data analysis

Mean values (30 per agar) from triplicate tests of each disc–strain combination were compared to target values and ranges in the EUCAST QC Tables v. 9.0, 2019 [10]. Each mean value was given a negative rating (0 to –5) as follows: 0 points if on target ±1 mm, –1 point if on target ±2 mm (but not ±1 mm), –3 points if > 2 mm from target but within range and –5 points if outside the QC range. For results outside range, it was also noted whether the results were above or below the range. The total rating per MH brand was calculated by summarizing the ratings given for each QC strain. The theoretical best rating of an agar was zero (all mean values on target ±1 mm) and the poorest rating –150 (all mean values outside range). For each MH brand, the percentage of individual zone diameters being on target ±1 mm and outside range was also calculated. The performance was considered 'excellent' when ≥99% of all zone diameters were within defined QC ranges and ≥70% on target ±1 mm for a MH brand.

Ethics considerations

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

Table 2
Antimicrobial agents, disc potencies and quality control strains

Antimicrobial disc	<i>Escherichia coli</i> ATCC 25922	<i>Pseudomonas aeruginosa</i> ATCC 27853	<i>Staphylococcus aureus</i> ATCC 29213	<i>Enterococcus faecalis</i> ATCC 29212	No. of tests per disc
Ampicillin 2 µg				●	3
Ampicillin 10 µg	●				3
Piperacillin-tazobactam 30–6 µg		●			3
Cefotaxime 5 µg	●				3
Cefoxitin 30 µg			●		3
Ceftazidime 10 µg	●	●			6
Imipenem 10 µg		●		●	6
Meropenem 10 µg	●	●			6
Ciprofloxacin 5 µg	●	●			6
Norfloxacin 10 µg			●	●	6
Gentamicin 10 µg	●	●	●		9
Gentamicin 30 µg				●	3
Tobramycin 10 µg		●			3
Erythromycin 15 µg			●		3
Tetracycline 30 µg			●		3
Tigecycline 15 µg	●		●	●	9
Linezolid 10 µg			●	●	6
Trimethoprim-sulfamethoxazole 1.25–23.75 µg	●		●	●	9
					90

Results

All 21 MH brands supported good growth of the four QC strains. Agar depths were within defined limits (3.5–4.5 mm) with mean values from 4.0–4.3 mm (Table 3). This was expected since all plates were produced at one site using identical Petri dishes, dispensing equipment and volumes.

Overall, there were 1890 zone diameter readings (90 per agar), of which 95% (1797/1890) were within QC ranges and 67% (1258/1890) were on target ± 1 mm. The accumulated rating for each brand ranged from -4 to -55 (Table 3). For agars from Bio-Rad, Biolife, Oxoid, Sigma (MH agar 2), BD (BBL MH II agar) and CRITERION, $\geq 99\%$ of zone diameter readings were within QC ranges and $\geq 70\%$ were on target ± 1 mm. The poorest ratings were for agar from Biolab and Merck (MH agar) with 10% (9/90) and 23% (21/90) of readings outside the QC ranges and 52% (47/90) and 44% (40/90) on target ± 1 mm, respectively.

Of all readings, 4.9% (93/1890) were outside the QC ranges, mostly related to testing of trimethoprim-sulfamethoxazole (25/189), aminoglycosides (25/315) and fluoroquinolones (15/252). For aminoglycosides and fluoroquinolones, all out-of-range results were above the QC ranges, whereas for trimethoprim sulfamethoxazole, 11/25 readings were above and 14/25 below the QC ranges. For results on individual agents and QC strains, see Tables S2–S6.

Agar pH and cation content

The pH values were within acceptable limits (7.2–7.4) for all but four brands (Table 3). The mean pH was above limits for agar from Biolab and Alpha Biosciences and below limits for HiMedia MH agar No. 2 and Merck MH agar according to CLSI. None of these four manufacturers recommended adjustment of pH during media preparation.

The concentrations of Ca, Mg, Zn, Mn and Fe ions in each of the MH brands are presented in Table 3. The cation content varied considerably between brands with concentrations ranging as follows (mg/L): Ca 6.8–43, Mg 4.0–64, Zn 0.36–7.8, Mn < 0.19 –23 and Fe < 0.34 –8.8. Clearly deviating levels were seen for Alpha Biosciences with Zn, Lab M with Mg, Merck MH Agar with Mn and HiMedia MH Agar with Fe.

Discussion

We evaluated the performance of 21 brands of dehydrated MH media available from 17 manufacturers. In a previous study, we evaluated antimicrobial discs from nine manufacturers and discovered wide variation in activity and quality [21]. The experiments were performed under standardized and blinded conditions and variations in test conditions were minimized. However, the performance varied between MH brands and many products showed results outside EUCAST QC criteria [10] for one or more antimicrobial agents. The brands with the poorest scores exhibited out-of-range results for several classes of agents and the use of these media will create major problems in EUCAST disc diffusion testing. There were also major differences in cation content and pH between the brands.

High concentrations of divalent cations, primarily calcium and magnesium, can decrease the activity of aminoglycosides and tetracyclines [14,15]. The ISO standard states that Ca and Mg concentrations in MH agar should be at a level that provides zones within the acceptable range for gentamicin with *Pseudomonas aeruginosa* ATCC 27853. Only one brand, HiMedia MH agar No. 2, produced zone diameters above the QC limits for this combination. This was also the product with the lowest concentration of both Ca and Mg. However, eight other MH brands, with varying concentrations of Ca and Mg, showed results above the QC range for gentamicin with *Enterococcus faecalis* ATCC 29212.

The zinc concentration in agar can affect testing with carbapenems. This can be controlled with *P. aeruginosa* ATCC 27853 and imipenem [16]. Agar from Alpha Biosciences had very high concentrations of zinc, but despite this results were inside QC ranges. The concentration of manganese has been shown to influence the *in vitro* activity of glycolcyclines and high Mn content is associated with false resistant interpretations [17]. Merck MH agar had an extremely high Mn content (23 mg/L) and this brand exhibited tigecycline zones below QC criteria. For iron, no acceptable limits have been published.

High content of thymine and thymidine in agar can inhibit the activity of sulphonamides and trimethoprim in *in vitro* testing [18,19]. The ISO Technical Specification [6] recommends low levels of thymidine (Table 1), as shown by a clear zone of inhibition within

Table 3
Results for 21 different brands of Mueller-Hinton (MH) dehydrated media for disk diffusion testing.

Total rating ^a	MH agar brand	Disc diffusion results				Cation content (mg/L) ^c					pH	Agar depth (mm)
		% zones on QC target ± 1 mm	% zones outside QC range	Agents ^b outside range, high	Agents ^b outside range, low	Ca	Mg	Zn	Mn	Fe		
-4	Bio-Rad MH Agar	86	0			43	9.6	0.47	<0.21	0.45	7.28	4.1
-10	Biolife MH Agar II	81	1.1	TS		43	19	0.53	<0.21	0.68	7.30	4.1
-10	Oxoid MH Agar	78	1.1	TS		24	15	0.39	<0.21	0.92	7.23	4.3
-11	Sigma MH Agar 2	81	0			19	8.4	0.50	<0.21	0.63	7.25	4.3
-12	BD BBL MH II Agar	73	0			21	20	0.89	<0.21	0.75	7.35	4.1
-12	CRITERION MH Agar	71	0			35	11	0.43	<0.21	0.59	7.31	4.1
-13	BD Difco MH Agar	70	3.3	AM		18	7.0	0.69	<0.21	0.65	7.36	4.1
-14	Alpha Biosciences MH Agar	71	3.3	FQ		40	6.7	7.8	<0.21	0.77	7.51	4.1
-17	E&O Labs MH Agar	82	8.9	CA/FQ/AM	TS	23	14	0.62	<0.21	0.66	7.37	4.2
-18	Sigma MH Agar	57	3.3	CS		19	8.5	0.54	<0.21	0.48	7.27	4.2
-20	HiMedia MH Agar	56	0			15	9.3	0.36	<0.21	8.8	7.30	4.2
-21	bioMérieux MHE Agar	64	3.3	TS		40	13	2.0	<0.21	0.57	7.20	4.1
-22	Acumedia MH Agar	63	3.3	AM		28	12	2.9	<0.21	0.65	7.21	4.2
-24	Remel MH Agar	64	6.7	AM	TS	27	17	1.1	<0.21	0.44	7.26	4.3
-25	Lab M MH Agar	69	6.7	AM	TS	14	64	0.52	<0.21	0.84	7.28	4.1
-25	Merck MH Agar acc. to CLSI	66	6.7	AM/TS		31	15	2.0	<0.21	0.54	7.16	4.0
-27	Mast MH Agar	59	8.9	CA/FQ	TS	8.8	7.7	0.61	<0.21	0.62	7.28	4.2
-31	Sifin MH Agar	60	6.7	AM/TS		29	15	1.9	<0.21	0.47	7.27	4.1
-32	HiMedia MH Agar No. 2	50	6.7	CA/AM		6.8	4.0	0.57	<0.21	1.3	7.18	4.0
-40	Biolab MH II Agar	52	10	PC/MA/TE	TS	17	38	0.56	<0.21	0.81	7.63	4.1
-55	Merck MH Agar	44	23	CS/CA/FQ/AM	TE	7.4	4.2	0.66	23	<0.34	7.34	4.1

^a Based on how mean values (30 per agar) from triplicate tests of four QC strains relates to the respective QC criteria (EUCAST QC Tables v. 9.0): 0 points if mean value on target ± 1 mm. -1 point if mean value on target ± 2 mm (but not ± 1 mm). -3 points if mean value > 2 mm from target but within range. -5 points if mean value outside range.

^b **Antimicrobial agents:** PC = Penicillins (ampicillin, piperacillin-tazobactam). CS = Cephalosporins (cefotaxime, cefoxitin, ceftazidime). CA = Carbapenems (imipenem, meropenem). FQ = Flouroquinolones (ciprofloxacin, norfloxacin). AM = Aminoglycosides (gentamicin, tobramycin). MA = Macrolides (erythromycin). TE = Tetracyclines (tetracycline, tigecycline). TS = Trimethoprim-sulfamethoxazole.

^c For MH requirements in DIN, FDA, ISO and WHO, see Table 1.

QC ranges for trimethoprim-sulfamethoxazole with *E. faecalis* ATCC 29212. The content of thymidine was not analysed in this study, but trimethoprim-sulfamethoxazole was the agent with most out-of-range results. Five brands exhibited results below the QC limits for *E. faecalis* ATCC 29212; Biolab, E&O Labs, Lab M, Mast and Remel.

FDA [8] and DIN [9] do not provide criteria for content of cations or thymidine in MH agar. The WHO [7] has published limits for Ca and Mg (Table 1) but these have not been updated since 1977 and none of the 21 tested products had concentrations within these limits. Attempts have been made to manipulate media by supplementing calcium and magnesium to meet performance criteria. However, the cation content of MH agar is a complex system and adding one cation can cause a shift between soluble and insoluble phases for others [23]. The autoclaving process can also affect the proportion of cations in agar [23]. Neither CLSI nor EUCAST recommend supplementation of MH agar with cations.

The pH of the media is known to influence testing of macrolides, tetracyclines and aminoglycosides. The brand with the most deviating pH, MH agar from Biolab (pH 7.63), exhibited results out of QC ranges both for erythromycin and tigecycline with *S. aureus* ATCC 29213. This agar was also one of the brands with poorest overall rating.

This evaluation was performed with in-house produced plates only. This was intentional to reduce variation related to commercial preparation, transportation, age and shelf life of pre-poured agar plates. Previous studies have reported variation in performance between MH manufacturers and between lots from the same manufacturer [24,25], but to our knowledge a large-scale study like this has not been performed. We did not examine lot-to-lot reproducibility but, in our experience, the variation between brands is larger than the variation between lots. The manufacturers were not informed about the evaluation and no attempts were made to investigate whether the 21 powders were of unique origin. For logistical reasons we had to limit the testing to 18 antimicrobial discs, carefully chosen to represent several antimicrobial classes and for their relevance to many species.

EUCAST [10] and CLSI [11] have published zone diameter QC criteria for manufacturers and users to evaluate the performance of discs and MH media. When QC strains and disc contents are identical, the EUCAST and CLSI criteria are identical except CLSI does not list target values, only ranges. The range is set to allow for unavoidable random variation in the laboratory and the target value to control systematic error, both in the laboratory and in the manufacturing of discs and media.

For in-house production of MH plates, laboratories should follow the manufacturers' recommendations, ensure a correct agar depth and pH and control the performance against EUCAST QC criteria. In-house prepared plates should be stored at 4–8 °C. Drying of plates, storage conditions and shelf life should be determined locally as part of the quality assurance programme [20]. Plates in this study were fresh and produced in-house and results cannot necessarily be compared with commercially produced and distributed plates of varying age. We encourage laboratories to investigate and report any media-related problems to the manufacturer. Breakpoints by EUCAST and CLSI were determined under conditions where discs and agars of the highest standard were used and QC criteria were met. The use of MH agar of poor quality will affect susceptibility test results and may lead to clinical isolates being reported as false susceptible or false resistant. Laboratories should be aware of these problems and include aspects on quality of discs and media in the procurement process. Mueller-Hinton media should meet the requirements in the ISO Technical specification [6] and the QC criteria published by EUCAST [10] and CLSI [11]. Several of the media with poor results in this study would have failed if these criteria had been used.

Conclusions

To obtain accurate and reproducible disc diffusion results, adherence to the standardized methodology and the use of media and antimicrobial discs of high quality is crucial. If any of these

components fail, the test fails. The formula for MH agar is described in standards from ISO, WHO, FDA and DIN; however, these are not in full agreement. Also, some of the components are of biological origin, which will cause variation in the medium. Components known to affect antimicrobial activity may, even when standardized, only be controlled by performance, *i.e.* measurement of inhibition zone diameters versus QC strains.

This study evaluated the performance and content of dehydrated MH media from several manufacturers worldwide. Six of 21 investigated MH brands showed excellent performance with EUCAST criteria, whereas other brands exhibited significant problems, partly explained by differences in cation content and pH. Both manufacturers and users must evaluate the performance of discs and media and confirm that QC criteria published by EUCAST or CLSI are met. The target values published by EUCAST provide additional support to manufacturers in the development of discs and media.

Author contributions

J. Åhman has performed the antimicrobial susceptibility testing. J. Åhman, E. Matuschek and G. Kahlmeter have planned the study and analysed and evaluated the results. All of the authors have contributed to writing the manuscript.

Transparency declaration

The study was not sponsored by the manufacturers of discs or media. G. Kahlmeter has previously consulted for Oxoid Ltd on technical matters, but not after 2016. This work was funded by the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) through its regular support of the development of EUCAST methodology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cmi.2020.01.018>.

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