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Research Article

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In Search of the Truth about the Quality of Mueller Hinton Agar and Tested Antimicrobial Discs

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Abstract *Objectives*: The main goal of the provided experiments was to test the influence of the used in the Disc Diffusion Method (DDM) Mueller Hinton agar (MHA) and to establish its reflection on the interpretation of the antimicrobial test.
Methods: This study is based on developed microbiological methodology and outworked protocol for qualification of the Mueller Hinton agar when it is used in Disc Diffusion Method in order to obtain reliable and reproducible results on quality indicators for MHA, appointed by the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST).
Results: Conducted studies showed the influence of the used medium MHA or MHA II on the zones of inhibition of antimicrobial discs in DDM, which can impact the interpretation of antibiotic susceptibility of isolates. This kind of testing of the medium MHA gives also the opportunity to establish some deviations in the activity of the antimicrobials used in the production of antimicrobial discs. In serial experiments was encountered a problem with the activity of Gentamicin substance that affects the quality of produced batches antimicrobial discs with him.
Conclusions: The experiments established the influence of the used medium MHA or MHA II on the zones of inhibition of antimicrobial discs in DDM. The used protocol with microbiological testing was very helpful as for evaluation of the MHA and for establishment of antimicrobials activity deviations.

Keywords Mueller Hinton agar, Disc Diffusion Method, zones of inhibition, antimicrobial discs, Gentamicin

Introduction

The quality of the supplied Mueller Hinton Agar, is essential for proper interpretation of the results of antibiogram by isolates from clinical specimens. The medium is designed and intended exclusively for use exactly for testing antimicrobial sensitivity of microorganisms. In this regard are set stringent standards for antimicrobial susceptibility testing on Disc Diffusion Method as well, according to CLSI [1], also according to EUCAST[2], which must be met in order not to report variations in the interpretation of the zones of inhibition[3,4]. These are identified as critical indicators for their impact on different antibiotics. Such requirements are related to thickness of the spilled in the plate medium, as well at pH of the MHA, content of thymine and thymidine, and also variation in the amount of bivalent cations (Ca^{2+}, Mg^{2+}). The first two parameters were fixed values, but for last two was mentioned only that the variation in their quantity will affect the size of the zones of inhibition of specific groups cited antibiotics and so can affect the interpretation of the antibiogram results. When performing routine quality control of each produced batch MHA should be established its suitability for use in antimicrobial susceptibility testing of isolates, observing

the requirements of the EUCAST [5].

Materials and Methods

Materials and Equipment

The study included MHA, produced from "Hi Media" and supplied by "BB-NCIPD" and MHA II, production of "BBL", spilled in plastic plates in "BB-NCIPD" according to the requirements of CLSI and EUCAST in a layer with 4 mm thickness. The test required 8 plates of each type of MHA. Were conducted parallel studies at the developed indirect method of medium MHA "Hi Media" lot N 215 790 and dehydrated media MHA II "BBL" lotN3084480, prepared according to the manufacturer's instructions and spilled under the same conditions of good manufacturing practices, equipment and personnel. The test was conducted in January 2016.

Were used antimicrobial discs in shelflife: Gentamicin 10 μg / disc, "Bio Lab", "BBL", "HiMedia" and "BB-NCIPD"; Tobramycin 10 μg / disc, "Bio Lab" and "BB-NCIPD"; Erythromycin 15 μg / disc, "BB-NCIPD"; Tetracycline 30μg / disc, "BB-NCIPD"; Doxycycline 30μg / disc, "BB-NCIPD"; Sulfamethoxazole / Trimethoprim 23.75 / 1.25μg / disc "BB-NCIPD".

The control strains used in the experiment were: *P. aeruginosa ATCC 27853, S. aureus ATCC25923, S. aureus ATCC 29213, E. coli ATCC 25922, E. faecalis ATCC29212.*

For standardization to turbidity 0.5 MF of the inoculum "DENSILAMETER II" and Vortex mixerwere used. Petri dishes with antibiograms were incubated in incubator "Memmert" at 35º C. To measure the pH of MHA and MHAII pH meter "Metrohm 744" was used.

Methodology

Preliminary preparation for quality control of MHA in a petri

Seven plates with MHA from each manufacturer were placed to control of sterility at 35º C for 24 hours. The eighth petri with MHA was placed in refrigerator for 24 hours and in the following day after tempering to 25° C was used for the direct measurement of a pH value with the surface electrode of the pH meter.

The interpretation of results is made according to theoretical instructions in CLSI M100-S17 [6]. Selected for analysis under critical indicators were representative types antimicrobial discs of the respective groups of antibiotics, shown in Table 1, 2, 3 and 4.

All of the used antimicrobial discs in the developed microbiological method and control strainsare presented in Table 1.

Table 1: Required to conduct the entire test antimicrobial discs and placing them on the corresponding control microorganism.

Reference strain	Antimicrobial disc
P. aeruginosa ATCC 27853	Gentamicin 10μg Tobramycin 10μg Ciprofloxacin 5μg Clindamycin 2μg
S. aureus ATCC 25923	Erythromycin 15μg Sulfamethoxazole / trimethoprim 23.75 / 1.25 μg Tetracycline 30μg
E. coli ATCC 25922	Doxycycline 30μg Sulfamethoxazole / Trimethoprim 23.75 / 1.25 μg
E. faecalis ATCC 29212	Sulfamethoxazole / Trimethoprim 23.75/1.25 μg

A disc with sulfamethoxazole / trimethoprim 23.75 / 1.25 μg was placed moreover on plate with *E. faecalis* ATCC 29212 also on plates with inoculations of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (as required by CLSI M 100) in order to identify deviations from the eligible zones on the reference strains at routine quality control of the disc.

The discs determined for each reference strain are placed always on two plates, to obtain a statistically significant result, by taking into account at least two zones of each type of disc on a reference strain. The established zones of inhibition are reflected in the tables as an arithmetic mean.

Variation in the content of bivalent cations / basicly calcium and magnesium

The increased content of bivalent cations reduces the diameter of the zones of inhibition of discs with aminoglycosides, and tetracyclines on strains *Pseudomonas aeruginosa*. The reduced content of bivalent cations leads to an inconsistent large zones of inhibition.

Table 2: Interpretation of the zones of inhibition obtained from Antimicrobial discs required for identifying any irregularities in critical indicator "content of divalent cations"

Indicator: content of Ca^{2+} and Mg^{2+}		
Antimicrobial Disc	**Zone greater than the upper limit**	**Zone less than the lower limit**
gentamicin 10μg, tobramycin10μg tetracycline 30μg, doxycycline30μg,	the content of Ca^{2+} and Mg^{2+} is too low	the content of Ca^{2+} and Mg^{2+} is too high

Interpretation of variations in pH according to established by the CLSI standards (7.2 to 7.4)

• At pH value lower than 7.2 antimicrobial substances from the group of aminoglycosides, quinolones and macrolides would be inactivated, while other agents (tetracycline) become more active, which is manifested in larger zones of inhibition.

• At pH value higher than 7.4 could be expected the opposite effect.

Table 3: Interpretation of the zones of inhibition obtained from Antimicrobial discs needed for identifying any irregularities in the critical indicator pH

Indicator: pH		
Antimicrobial Disc	**Zone greater than the upper limit**	**Zone less than the lower limit**
ciprofloxacin 5μg clindamycin 2μg erythromycin15μg gentamicin 10μg, tobramycin10μg	pH is higher than 7.4	pH is less than 7.2
tetracycline 30μg, doxycycline30μg	pH is less than 7.2	pH is higher than 7.4

The inappropriate content of thymine or thymidine affects at the inhibitory effect of sulfonamides and trimethoprim.

- The increased content of thymine or thymidine may have an opposite effect on the inhibitory activity of the sulphonamides and trimethoprim, resulting in giving smaller and less distinct zones or totally lacking such. This will give a wrong interpretation of the results for resistance.
- For properly balanced against the content of thymine and thymidine media clear zones of inhibition with a diameter ≥ 20 mm at placed disc with trimethoprim / sulfamethoxazole on *Enterococcus faecalis* ATCC 29212 have to be observed.
- For ineligible result according this indicator the media MHA will be with missing zones of inhibition or the zone will be<20 mm.

Table 4: Interpretation of the zones of inhibition obtained from Antimicrobial discs needed for identifying any irregularities in critical indicator "content of thymine and / or thymidine."

Indicator: content of thymine and / or thymidine		
Antimicrobial disc	**Zone greater than 20 mm**	**Zone less than 20 mm**
Sulfamethoxazole / trimethoprim 23.75 / 1.25μg	The medium is with corresponding content of thymine and / or thymidine	The medium hastoo high content of thymidine

Results

Before starting the test at the developed protocol to characterize medium MHA, the pH of the plates, spilled with agar from the two batches tested media was measured directly with a surface electrode, establishing compliance with the norm.

Due received a different interpretation at discs gentamicin and tobramycin in the study were included as antimicrobial discs produced by the company "Bull Bio-NCIPD" and discs offered by "Bio Lab". The results of a comparative study are presented in Tables 5 and 6.

Table 5: Results from testing the properties of MHA "Hi Media" lot N 215 790 by the indirect microbiological method

Antimicrobial discs, Producer	Microbial strain ATCC	According ….. Norm (mm)	Zone (mm)	Conclusion
Gentamicin 10μg/disc, "BB-NCIPD"	*P.aeruginosa ATCC 27853*	CLSI:16-23 EUCAST: 17-23	23.5	Ca^{2+} and Mg^{2+}: correct pH /7.2-7.4/: correct
Gentamicin 10μg/disc, "Bio Lab"	*P.aeruginosa ATCC 27853*	CLSI:16-21	21	Ca^{2+} and Mg^{2+}: correct pH /7.2-7.4/: correct
Tobramycin 10μg/disc, "BB-NCIPD"	*P.aeruginosa ATCC 27853*	CLSI: 19-25	23.5	Ca^{2+} and Mg^{2+}: correct pH /7.2-7.4/: correct
Tobramycin 10μg/disc, "Bio Lab"	*P.aeruginosa ATCC 27853*	CLSI: 19-25 EUCAST: 20-26	21	Ca^{2+} and Mg^{2+}: correct pH /7.2-7.4/: correct
Erythromycin 15μg/disc, "BB-NCIPD"	*S.aureus ATCC25923*	CLSI: 22-30	30.5	pH /7.2 -7.4/: correct
	S.aureus ATCC 29213	EUCAST: 23-29	28.5	
Tetracycline 30μg/disc, "BB-NCIPD"	*E.coli ATCC 25922*	CLSI: 18-25	26	
	S.aureus ATCC 29213	EUCAST: 23-31	30.5	Ca^{2+} and Mg^{2+}: correct
Doxycycline 30μg/disc, "BB-NCIPD"	*E.coli ATCC 25922*	CLSI:18-24	24	Ca^{2+} and Mg^{2+}: correct
Sulfamethoxazole/Trimethoprim 23.75/1.25μg/disc "BB-NCIPD"	*E.faecalis ATCC29212*	CLSI: > 20 EUCAST:26-34	32	Thymidine: correct

According to the used protocol, developed to characterize MHA for testing of antimicrobial discs of the two companies on plates with the medium MHA, lot N 215 790 production of "Hi Media" it was found that the zones of inhibition for all discs were in norm which covers the quality criteria of the medium according to the requirements by indicators.

Table 6: Results of testing the quality of MHA II "BBL" lot N3084480 through the indirect microbiological method

Antimicrobial discs , Producer	Microbial strain ATCC	According ….. Norm (mm)	Zone (mm)	Conclusion
Gentamicin 10μg/disc, "BB-NCIPD"	*P.aeruginosa ATCC 27853*	CLSI:16-23 EUCAST: 17-23	25.5	Ca^{2+} and Mg^{2+}: incorrect pH /7.2-7.4/: incorrect
Gentamicin 10μg/disc, "Bio Lab"	*P.aeruginosa ATCC 27853*	CLSI:16-21	22.5	Ca^{2+} and Mg^{2+}: incorrect pH /7.2-7.4/:

				incorrect
Tobramycin 10μg/disc, "BB-NCIPD"	*P.aeruginosa ATCC 27853*	CLSI: 19-25	28.5	Ca^{2+} and Mg^{2+}: incorrect pH /7.2-7.4/: incorrect
Tobramycin 10μg/disc, "Bio Lab"	*P.aeruginosa ATCC 27853*	CLSI: 19-25 EUCAST: 20-26	23.5	Ca^{2+} and Mg^{2+}: correct pH /7.2-7.4/: correct
Erythromycin 15μg/disc, "BB-NCIPD"	*S.aureus ATCC25923*	CLSI: 22-30	29	pH /7.2-7.4/: correct
	S.aureus ATCC 29213	EUCAST: 23-29	28	
Tetracycline 30μg/disc, "BB-NCIPD"	*E.coli ATCC 25922*	CLSI: 18-25	24	
	S.aureus ATCC 29213	EUCAST: 23-31	24	Ca^{2+} and Mg^{2+}: correct
Doxycycline 30μg/disc, "BB-NCIPD"	*E.coli ATCC 25922*	CLSI:18-24	23	Ca^{2+} and Mg^{2+}: correct
Sulfamethoxazole/Trimethoprim 23.75/1.25μg/disc "BB-NCIPD"	*E.faecalis ATCC29212*	CLSI: > 20 EUCAST:26-34	31.5	Thymidine: correct

The comparison of the properties of the MHA and MHA II is visualized in Figure 1.

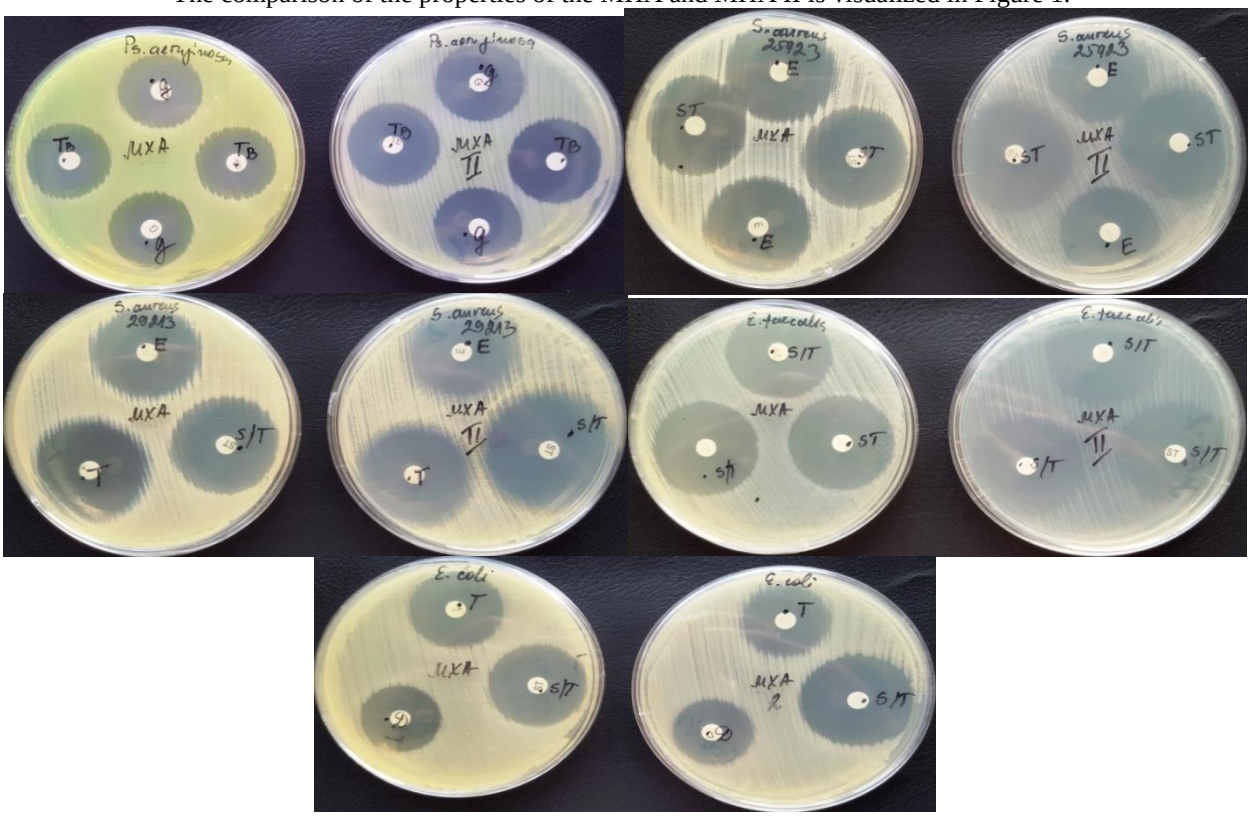

Figure 1: Comparison between the properties of both type Muelller Hinton Agar - MHA and MHA II. On the left side it is petri with MHA and on the right side– petri with MHA II. (Legend: S/T – Sulfamethoxazole / Trimethoprim , G- Gentamicin .Tb- Tobramycin, E- Erythromycin, T - Tetracycline D- Doxycycline.)

When testing the same antimicrobial discs on plates with medium MHA II "BBL" lot №3084480 it was taken account of the change in the interpretation of two types of disc loaded with gentamicin and tobramycin, which are representatives of aminoglycoside antibiotics. At discs with gentamicin, produced bythe two companies was established according to the protocol discrepancy in two indicators: the content of Ca^{2+} and Mg^{2+} and pH (norm: 7.2 -7.4).At the discs loaded with tobramycin discrepancy was once again taken into account under the same two indicators, but only on discs that are produced by "BB-NCIPD". Those production of "Bio Lab", were in norm.Taking into account that the pH is established in a norm by direct measurement of the media with surface electrode, reporting of discrepancy in this indicator is embarrassing. In search of the reason for this, the study was expanded to focus on testing as required by CLSI for discs loaded with gentamicin in shelf life offered by four different companies (Figure 2, Table 7).

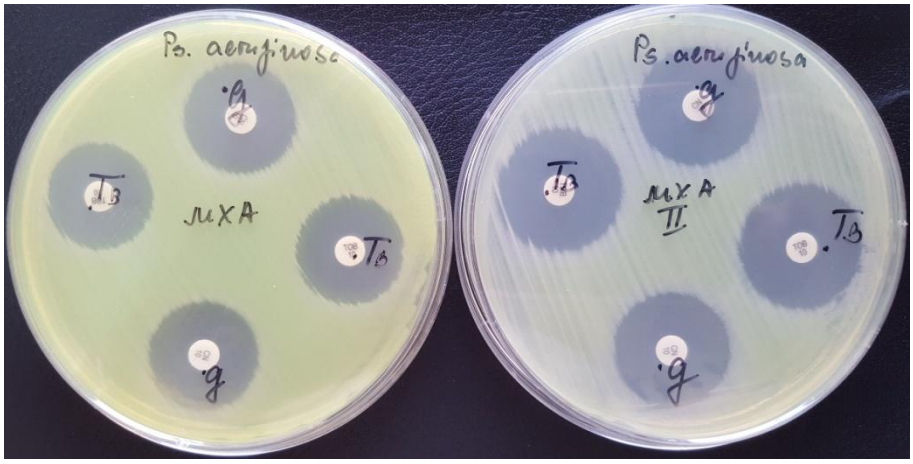

Figure 2: Comparison between the properties of MHA and MHA II with gentamicin discs and tobramicin discs, produced by other companies.

Table 7: Zones of inhibition according to the standards for quality control of antimicrobial discs CLSI, production of 4 different companies on medium of "HiMedia".

Control strain according CLSI	**Zone of inhibition of Gentamicin 10 µg / disc (mm)**						
	Norm according CLSI	"BBL", lot N 5273907, Exp. 31.10.2019	"BBL", lot N 5026760, Exp. 28.02.2019	"BioLab", lot N 140725, Exp. 03.2017	"BioLab", lot N 141226B, Exp. 08.2017	"BB - NCIPD" , lot N 460116, Exp. 12.2016	"HiMedia", lot N 0000262136, Exp. 04.2018
E.coli ATCC 25922	**19-26**	14.5	14.5	14	13.5	18.5	18
S.aureus ATCC 25923	**19-27**	18	18	18	16.5	22	22
P. aeruginosa ATCC 27853	**17-23**	18.5	19	20.5	15.19	24	25.5

Discussion

A deviation in the zones of inhibition was observed on the strain *E. coli* ATCC 25922 at all tested batches of discs with gentamicin of the 4 companies. It should be noted that close to the lower limit of norm for the strain are discs, production of "BB -NCIPD" and "HiMedia", while those of the batches of "BBL" and "BioLab", have a much greater deviation in direction downwards from the norm. The same trend was observed also at testing on *S. aureus*

ATCC 25923 and *P. aeruginosa* ATCC 27853, although the discs of all companies were in norm for corresponding strains. In search of the truth about the problem was raised the issue for the used at the quality control microbial strains. A testing with strain *E. coli* ATCC 25922 taken from the Reference Laboratory "Control and monitoring of antibiotic resistance" at NCIPD was also conducted and the resulting zones of inhibition were similar.

It is noteworthy that in a study of EUCAST the quality of antimicrobial discs on 9 manufacturers conducted in two consecutive years only in 2014 was tested the disc with gentamicin from 8 companies. In 2015 in the monitoring of this disc participate only 4 companies [7]. BD and Oxoid are not among them, despite that they had participated in the previous monitoring. This raises the suspicion that the problem with the disc gentamicin really exists.

Hence, the question for adequate load with an antibiotic of the discs was raised, which in turn is mainly determined by the activity of the used antimicrobial substance.

In microbiological testing of batches of ampoules with injectable gentamicin from 3 different pharmaceutical companies, and 3 different batches substances gentamicin from "Sigma - Aldrich" (lots №№SLBK9711V; SLBG7734V; SLBM9736V), the established activity was below 50% of the result declared by the manufacturer. This raises the question of the necessity of centralized control of the used in praxis antibiotics and in particular on the critical indicator for them - the activity of the antimicrobial substances. It is well known that antibiotics have different activity in vivo than in vitro, especially in sputum[8].The use of gentamicin is intravenous and applicable in situations when we are searching for synergism or at life threatening infections [9,10].

Conclusion

Conducted studies showed the influence of the used medium MHA or MHA II on the zones of inhibition of antimicrobial discs in DDM, which can impact the interpretation of antibiotic susceptibility of isolates.

The microbiological testing was very helpful also for the establishment of antimicrobials activity deviations as during the test a problem with the activity of Gentamicin substancethat affects the quality of the produced batches of antimicrobial discs with it was encountered.

Manufacturers bear the responsibility for not releasing lots of antimicrobial discs with low activity for sale, as this could affect the determining the antibiotic sensitivity of the tested bacterial isolates and at the end of the chain it would affect the usefulness of the prescribed treatment to the patient.

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Declarations

Funding: No funding. This study was conducted as part of our routine work.

Competing Interests: None to declare.

Ethical Approval: Not required

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