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I J N R E

Influence of culture media on the Kirby–Bauer method for testing antibacterial activity against *Pseudomonas aeruginosa* (HO80)

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Abstract:

We investigated in this study the effect of different culture media on antibiotic susceptibility testing of *Pseudomonas aeruginosa* HO80 with the Kirby–Bauer (agar-diffusion) method by measuring the diameters of inhibition zones. We observed that the generally used culture medium, Mueller-Hinton agar (MHA*), could be replaced by many other culture media, such as Hektoen agar (HKA*), Bennett's agar (BAM) and Trypticase Soy Agar (TSA*) media in order to realize antibiograms used in medical and research laboratories for testing *Pseudomonas aeruginosa*.

Keywords: Culture medium; Antibiotic; *Pseudomonas aeruginosa* HO80; Antibiogram; Kirby–Bauer method.

Résumé:

Nous avons étudié dans ce travail l'effet des milieux de culture sur la sensibilité aux antibiotiques vis-à-vis de *Pseudomonas aeruginosa* HO80 avec la méthode de Kirby–Bauer (diffusion sur disque) en mesurant les diamètres des zones d'inhibition. Les résultats obtenus ont montré que le milieu de culture Mueller-Hinton gélosé (MHA*) pouvait être remplacé par de nombreux autres milieux, tels que Hektoen agar (HKA*), Bennett's agar (BAM) et Trypticase Soy Agar (TSA*) afin de réaliser des antibiogrammes utilisés dans les laboratoires médicaux et de recherche pour tester *Pseudomonas aeruginosa*.

Mots clés: Milieu de culture ; Antibiotique ; *Pseudomonas aeruginosa* HO80 ; Antibiogramme ; Méthode de Kirby–Bauer.

1. Introduction

Agar-diffusion method named also Kirby–Bauer test, disk-diffusion or agar dilution method has been widely used for antibacterial susceptibility testing of environmental, medical and/or veterinary bacterial strains (Perreten et al., 1997, 1998; Butaye et al., 2000; Huys et al., 2002).

The solid culture medium provides an appropriate matrix to allow reproducible and homogeneous diffusion of the antibiotic which minimizes possible interactions between culture medium components and the tested antibiotics (Huys et al., 2002). The Kirby–Bauer test was recommended by the Antibiogram Committee of the French Microbiology Society (ACFMS) and also by the Algerian Network for Monitoring the Resistance of Bacteria to Antibiotics (ANMRBA). Huys et al. (2002) indicated that Mueller-Hinton (Syn. Müller-Hinton) agar is considered to exert only limited antagonistic effects against the majority of test reagents. Consequently, this culture medium is the unique recommended culture medium to develop antibiograms worldwide.

Pseudomonas aeruginosa affects generally the immunocompromised, but also infect in some cases the immunocompetent persons. This microorganism is an opportunistic pathogen as responsible of infections of many diseases such as cystic fibrosis and traumatic burns. Treatment of *P. aeruginosa* infections is difficult due to its natural resistance to many antibiotics.

The objective of this work is to evaluate the performance of many culture media as a substitute for the Mueller-Hinton agar medium in determining the antibacterial sensitivity of *Pseudomonas aeruginosa* (HO80) by Kirby–Bauer test. Using the inhibition zone diameter as a quantitative performance parameter, ten (10) complex culture media were evaluated for eight (8) antibacterial agents.

2. Materials and Methods

2.1. Bacterial strain

Pseudomonas aeruginosa (HO80): a common encapsulated, Gram-negative, rod-shaped bacterium that can cause disease in humans [*Pseudomonadaceae*, *Pseudomonadales*, *Gammaproteobacteria*, *Proteobacteria*] was provided from the hospital of Ouargla (Algeria). Bacterial identification was checked by chromogenic medium (chromagar).

2.2. Culture media and antibiotics

The antibiotic sensitivity profile was determined by using ten (10) undefined (complex) solid media: Mueller-Hinton agar (MHA*) purchased from Imen Lab.; Nutrient Agar (NA*) purchased from Dimed; Nutrient Agar (NA) composed of 5 g/L meet extract (Biochem Chemopharma), 10 g/L bacteriological peptone (Conda pronadisa), 5 g/L NaCl (TM Media) and 20 g/L agar (Biochem Chemopharma), the pH of the medium was adjusted to 7.0 before sterilization; Hektoen agar (HKA*) purchased from Dimed; Trypticase Soy Agar (TSA*) purchased from Conda pronadisa; Chapman medium (called also Mannitol salt Agar: MSA*) purchased from Dimed; International Streptomyces Project (ISP2) composed of 4 g/L malt extract (Conda pronadisa), 4 g/L yeast extract (Conda pronadisa), 4 g/L glucose (VWR Prolabo) and 20 g/L agar (Biochem Chemopharma), the pH of the medium was adjusted to 7.0 before sterilization; Bennett's agar medium (BAM) composed of 10 g/L glucose (VWR Prolabo), 2 g/L bacteriological peptone (Conda pronadisa), 1 g/L yeast extract (Conda pronadisa), 1 g/L meat extract (Biochem Chemopharma) and 20 g/L

agar (Biochem Chemopharma), the pH of the medium was adjusted to 7.0 before sterilization; Potato dextrose agar (PDA*) which generally used to cultivate micro-fungi, purchased from Conda pronadisa; and Sabouraud Agar (SA, generally used for micro-fungi) composed of 20 g/L glucose (VWR Prolabo), 10 g/L bacteriological peptone (Conda pronadisa) and 20 g/L agar (Biochem Chemopharma), the pH of the medium was adjusted to 7.0 before sterilization.

Pseudomonas aeruginosa (HO80) was evaluated against eight antibacterial agent disks (all purchased from Himidia) were included in this study: Imipenem (IPM: 10 µg), Colistin (CL: 30 µg), Piperacillin (PIP: 100 µg), Aztreonam (ATM: 30 µg), Ticarcillin + Clavulanic acid (TCC: 75+10 µg), Gentamicin (GEN: 10 µg), Rifampicin (R: 5 µg) and Ceftazidime (CAZ: 30 µg). The antibacterial activity was estimated by measuring the diameter of the inhibitory zone. Inhibition zones were measured by a ruler, and each value is the average of three trials.

2.3. Agar overlay disk-diffusion testing

Susceptibility testing was based on the Kirby–Bauer (agar disk-diffusion) method. From fresh overnight grown culture of *P. aeruginosa* HO80 (20-24 h), many colonies were suspended in 10 mL of sterilized physiological water. The inoculum density of the liquid cultures was adjusted to a concentration of 0.5 McFarland (optical density ranging from 0.08 to 0.1) using a Dialab spectrophotometer.

Petri plates (90 mm in diameter) containing 20 mL of each culture medium were allowed to solidify for 20 min at RT (25 ± 2 °C). A cotton swab was used for inoculation. Antibiotic disks were placed onto the overlay dishes and all dishes were incubated for 24 h (at 37°C).

2.4. Statistical analysis

Data collected were statistically analyzed by factorial ANOVA and Tukey HSD test, using XLSTAT 2018 at the probability level $P < 0.05$. Means were compared to a control treatment (MHA* medium) using Dunett's bilateral test.

3. Results and Discussions

The results showed highly significant effects for factors (culture medium and antibiotic), as well as for their interaction ($P<0.0001$).

The influence of different culture media is summarized in Table 1 and Figure 1. The statistical analyses showed, on the nine culture media used, that *Pseudomonas aeruginosa* HO80 is very sensitive to Imipenem (inhibition zone ranging between 28.0 and 41.5 mm) and Aztreonam (inhibition zone ranging between 30.0 and 38.0 mm); and sensitive to Colistin (inhibition zone ranging between 11.0 and 22.0 mm) and Rifampicin (inhibition zone ranging between 10 and 18 mm). However, all other antibiotics (Piperacillin, Ticarcillin + Clavulanic acid, Gentamicin and Ceftazidime) are globally moderately sensitive (inhibition zone ranging between 14.0 and 31 mm). The obtained results indicated that *Pseudomonas aeruginosa* HO80 grow well on nine tested culture media, except Chapman medium (Mannitol salt Agar), which is generally used for halophilic bacteria such as *Staphylococcus aureus* and some *Bacillaceae*.

Table. 1. Inhibition zone diameters (in mm) obtained for each culture medium/antibiotic combination of *Pseudomonas aeruginosa* HO80.

	IPM	CL	PIP	ATM	TCC	GEN	R	CAZ
MHA*	36.0 ± 0.0	20.5 ± 0.5	26.0 ± 0.0	34.0 ± 0.0	24.0 ± 1.0	26.0 ± 0.0	10.0 ± 0.0	21.5 ± 0.5
NA*	41.5 ± 0.5	13.0 ± 1.0	24.0 ± 1.0	32.0 ± 0.0	25.0 ± 1.0	21.0 ± 1.0	10.0 ± 0.0	19.0 ± 1.0
NA	38.0 ± 2.0	15.5 ± 0.5	22.0 ± 2.0	30.0 ± 0.0	18.0 ± 1.0	22.0 ± 0.0	15.0 ± 1.0	16.0 ± 0.0
HKA*	28.0 ± 2.0	19.5 ± 1.5	28.5 ± 1.5	38.0 ±1.0	28.0 ± 0.0	22.0 ± 0.0	11.0 ± 1.0	25.0 ± 1.0
TSA*	34.5 ± 0.5	22.0 ± 2.0	20.0 ± 2.0	32.0 ± 2.0	22.5 ± 1.5	25.0 ± 1.0	10.0 ± 0.0	23.5 ± 0.5
ISP2	39.0 ± 1.0	13.0 ± 0.0	24.0 ± 4.0	30.5 ± 0.5	22.5 ± 0.5	15.0 ± 1.0	18.0 ± 1.0	18.0 ± 0.5
BAM	37.0 ± 1.0	11.0 ± 0.0	29.0 ± 1.0	38.0 ± 2.0	31.0 ± 1.0	14.0 ± 0.0	10.5 ± 0.5	21.0 ± 1.0
PDA*	38.0 ± 2	15.0 ± 0.0	23.0 ± 0.0	30.5 ± 0.5	19.0 ± 1.0	21.0 ± 1.0	11.0 ± 1.0	18.0 ± 0.0
SA	30.0 ± 0.0	16.5 ± 0.5	28.0 ± 2.0	30.0 ± 1.0	20.5 ± 1.5	21.0 ± 1.0	10.0 ± 0.0	18.0 ± 1.0

MHA: Mueller-Hinton agar; NA: Nutrient Agar; HKA: Hektoen agar; TSA: Trypticase Soy Agar; ISP2: International Streptomyces Project 2; BAM: Bennett's agar medium; PDA: Potato dextrose agar; SA: Sabouraud Agar; * : purchased ready to use. IPM: Imipenem (10 µg); CL: Colistin (30 µg); PIP: Piperacillin (100 µg); ATM: Aztreonam (30 µg); TCC: Ticarcillin + Clavulanic acid (75+10 µg); GEN : Gentamicin (10 µg); R : Rifampicin (5 µg) and CAZ : Ceftazidime (30 µg). Values shown are the average mean values ± standard deviation. No growth was observed on MSA* (Mannitol salt Agar = Chapman medium).

Based on statistical analysis we noticed that there is no significant difference between MHA* and HKA*, BAM media (based on both Dunett and Tukey HSD tests), or with TSA* medium (based only on Tukey HSD test) (Fig. 1). Consequently, the MHA* could be replaced by these three-culture media in medical and research laboratories. Furthermore, the antibiograms for each culture medium/antibiotic combination revealed that TSA* medium had similar inhibition zone for the majority of antibiotics. However, the NA medium showed the most significant difference among all culture media tested (Table 1).

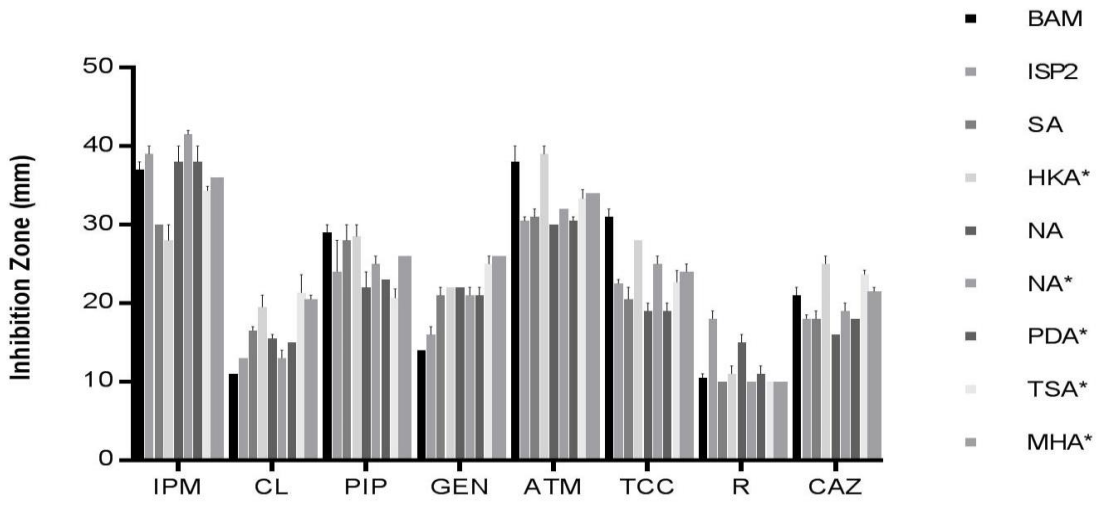

Fig. 1. Inhibition zone diameters (in mm) obtained for each culture medium/antibiotic combination of *Pseudomonas aeruginosa* HO80.

MHA: Mueller-Hinton agar; NA: Nutrient Agar; HKA: Hektoen agar; TSA: Trypticase Soy Agar; ISP2: International Streptomyces Project 2; BAM: Bennett's agar medium; PDA: Potato dextrose agar; SA: Sabouraud Agar; * : purchased ready to use. IPM: Imipenem (10 µg); CL: Colistin (30 µg); PIP: Piperacillin (100 µg); ATM: Aztreonam (30 µg); TCC: Ticarcillin + Clavulanic acid (75+10 µg); GEN : Gentamicin (10 µg); R : Rifampicin (5 µg) and CAZ : Ceftazidime (30 µg). Values shown are the average mean values ± standard deviation. No growth was observed on MSA* (Mannitol salt Agar = Chapman medium).

Table. 2. Significance levels of inhibition zone diameters (in mm) obtained for each culture medium/antibiotic combination of *Pseudomonas aeruginosa* HO80, compared to MHA* control.

	IPM	**CL**	**PIP**	**ATM**	**TCC**	**GEN**	**R**	**CAZ**
NA*	***	***	*ND*	*	*ND*	**	*ND*	*ND*
NA	*ND*	**	*	*ND*	**	*	**	***
HKA*	***	*ND*	*ND*	**	*	*	*ND*	*ND*
TSA*	*ND*	*ND*	***	*ND*	*ND*	*ND*	*ND*	*ND*
ISP2	*ND*	***	*ND*	*ND*	***	***	***	***
BAM	*ND*	***	*ND*	*	***	***	*ND*	*ND*
PDA*	*ND*	***	*ND*	*ND*	**	**	*ND*	*ND*
SA	***	*	*ND*	*ND*	*ND*	***	*ND*	*ND*

*MHA: Mueller-Hinton agar; NA: Nutrient Agar; HKA: Hektoen agar; TSA: Trypticase Soy Agar; ISP2: International Streptomyces Project 2; BAM: Bennett's agar medium; PDA: Potato dextrose agar; SA: Sabouraud Agar; * : purchased ready to use. IPM: Imipenem (10 µg); CL: Colistin (30 µg); PIP: Piperacillin (100 µg); ATM: Aztreonam (30 µg); TCC: Ticarcillin + Clavulanic acid (75+10 µg); GEN : Gentamicin (10 µg); R : Rifampicin (5 µg) and CAZ : Ceftazidime (30 µg). Values shown are the average mean values ± standard deviation.*

*Data showed the results of Tukey HSD test with significance levels: (P<0.0001 ***); (P<0.01 **); (P<0.05 *); (P>0.05 ND)*

Table. 3. Performance standards (in mm) for antimicrobial susceptibility testing used for *Pseudomonas aeruginosa* (Standardization of antibiograms for human and veterinary Medicine, 2011).

	R	**I**	**S**
IPM	≤ 13	14-15	≥ 16
CL	≤ 10	——	≥ 11
PIP	≤ 17	——	≥ 18
ATM	≤ 15	16-21	≥ 22
TCC	≤ 14	——	≥ 15
GEN	≤ 12	13-14	≥ 15
R	≤ 14	15-18	≥ 19
CAZ	≤ 14	15-17	≥ 18

R = Resistant, S = Susceptible, I = Intermediate.

Table. 4. Antibiotic susceptibility pattern of *Pseudomonas aeruginosa* HO80 (R = Resistant, I = Intermediate, S = Susceptible).

	IPM	CL	PI	ATM	TCC	GEN	R	CAZ
MHA*	S	S	S	S	S	S	R	S
NA*	S	S	S	S	S	S	R	S
NA	S	S	S	S	S	S	I	I
HKA*	S	S	S	S	S	S	R	S
TSA*	S	S	S	S	S	S	R	S
ISP2	S	S	S	S	S	S	I	S
BAM	S	S	S	S	S	I	R	S
PDA*	S	S	S	S	S	S	R	S
SA	S	S	S	S	S	S	R	S

MHA: Mueller-Hinton agar; NA: Nutrient Agar; HKA: Hektoen agar; TSA: Trypticase Soy Agar; ISP2 : International *Streptomyces* Project 2 medium; BAM: Bennett's agar medium; PDA: Potato dextrose agar; SA: Sabouraud Agar; * : purchased ready to use. IPM: Imipenem (10 µg); CL: Colistin (30 µg); PIP: Piperacillin (100 µg); ATM: Aztreonam (30 µg); TCC: Ticarcillin + Clavulanic acid (75+10 µg); GEN : Gentamicin (10 µg); R : Rifampicin (5 µg) and CAZ : Ceftazidime (30 µg).

Based on Table 3, we observed that, except Gentamicin (in Bennett's agar medium), Rifampicin (in Nutrient Agar and International Streptomyces Project 2 medium) and Ceftazidime (in Nutrient Agar), all other tested antibiotics showed no change in resistance profile by using different culture media. This finding confirmed that the usually used Mueller-Hinton medium could be switched by many other culture media in medical and research laboratories for antibiogram testing. The unique precaution should be taken in the case of Gentamicin, Rifampicin and Ceftazidime when tested in some culture media as indicated above. The same observation has been reported for Gentamicin in testing different culture media with Escherichia coli (Daoudi et al., 2017). The results obtained for Gentamicin, Rifampicin and Ceftazidime testing did not allow a clear interpretation.

It has been stated that the composition of culture media exercises an key effect on the antimicrobial susceptibilities of microorganisms (Pfaller et al., 1998; Arikan et al., 1999; Meletiadis et al., 2001). Garrod and Waterworth (1971) examined supplies of Mueller-Hinton medium from three different manufacturers and found considerable differences in their inhibitor content. This difference being probably attributable to the magnesium content of the agar used (Garrod and Waterworth, 1969). Furthermore, Garrod and Waterworth (1971) observed that the composition of medium affects very considerably the result obtained with *Pseudomonas aeruginosa*.

Agar-based methods are quick and easy and could be good choice (Fernández-Torres et al., 2006). Garrod and Waterworth (1971) described that the culture medium used should be free from inhibitors such as antibiotics (trimethoprim, tetracycline, sulfonamide, etc.).

Although the reference methods recommend the use of Mueller-Hinton medium for agar testing, other less-expensive media have also been used with success (Llop et al., 1999; Müller et al., 2001; Pfaller et al., 1998). In this study, we observed that it's not necessary to use only Mueller-Hinton medium to

produce antibiograms, and many other culture media could be used successfully to obtain the same results.

With the increase in the incidence of systemic bacterial infections and with the growing number of antibiotics needed for be tested, the interest in laboratory methods to guide the selection of antibacterial therapy has also increased. The reference method for *in vitro* tests of for determination of the antibacterial sensitivity of bacterial strains recommends the use of the Mueller-Hinton agar. However, while this is relatively a high-cost medium, the use of other culture media with low-cost medium could be widely used in microbiology.

4. Conclusion

it's necessary to mention that these data have been obtained under the same conditions of pH and temperature (7 and 37°C, respectively) for all culture used media; however, only one strain of *P. aeruginosa* (named HO80) has been used in this work, and this should be taken into consideration for any conclusion.

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