Al-Qadisiyah Journal of Agriculture Sciences
(QJAS)
| 2077-5822 |

Development of *in-vitro* Sensitivity Testing for Pathogenic Bacteria

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Submission Track
Received : 2/4/2017
Final Revision : 3/5/2017
Keywords
Susceptibility, MIC, antibiotic, medical laboratories technique.
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Abstract
A new method developed for *in-vitro* susceptibility test in medical laboratories consist of micro tubes or gloves containing dehydrated tryptic soya broth, 5% glucose, 0.1% bromothymol blue and one type of antibiotics (ampicillin, tetracycline and chloramphenicol) with critical concentration MIC (minimum inhibitory concentration) for susceptibility. Standard quality control strains of bacterial (*Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) suspension were adjusted to 0.5 McFarland turbidity standard (1×10^6 cell/mL) were used in inoculation the media and incubated two hours at 37 °C. The MIC of ampicillin against *E. coli*, *S. aureus*, and *P. aeruginosa* were 4, 32, and 256 µg/mL of the media for the bacteria respectively, while the MIC of tetracycline against bacteria were 512, 512 and 32 µg/mL respectively, the MIC of chloramphenicol were 512, 32 and 512 µg/mL, respectively. Where, the resistant bacteria to the antibiotics could grow and ferment glucose sugar producing a color change of the media from blue to yellow, while the sensitive bacteria do not grow or show no change in color. Our study result compared with common used antibiotic disk method obtaining similar results. This developed method characterized by fast (only two hours) and less cost in comparison to conventional technique. The new micro tube strip is highly stable (more than one year) with more sensitive in detection of variable pathogenic bacteria including standard bacteria strains compared with conventional technique.

Introduction

The clinical symptom of an infectious disease reflects the interaction of the pathogenic microorganism with the host. This interaction is affected by microbial virulence factors and the host immune status. The symptoms and signs are difference according to the site and severity of infection [1]. The diagnosis requires a composite of information including history, physical examination, radiographic findings, and laboratory data [2].

The determination of microbial susceptibility to antimicrobials is very important responsibility of the microbiology laboratory after microbial detection and isolation [3, 4]. The term susceptible means that the microorganism is inhibited by a concentration of antimicrobial agent that can be present in blood with the normally depended dose of the antimicrobial reagent and suggested that an infection occurred by this microorganism may be appropriately controlled with the antimicrobial agent. Microbial resistant indicates that the microorganism is resistant to concentrations of the antimicrobial agent that can be obtain with normal doses and implies that an microbial infection could not be successfully treated with this antimicrobial agent [5, 6].

Many bacteria have unpredictable susceptibilities to antimicrobial agents and their susceptibilities can be measured *in vitro* to help the choice of the most

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appropriate antimicrobial agent. The widely used susceptibility testing methods are the disk diffusion and broth agar dilution tests. The MIC (minimum inhibitory concentration) of a particular drug to a organism can be quantitatively determined *in-vitro* through the broth agar or dilution test. These testing methods have been standardized and the NCCLS (National Committee of Clinica1 Laboratory Standards) provides susceptibility test guidelines [6-8]. In this study we tried to improve new technique for susceptibility test of pathogenic bacteria to the antibiotics.

Materials and Methods

Measurement of MIC (Minimum Inhibitory Concentration)

Stock solution: Ampicillin (Aldrich/ Sigma) (50 mg/mL), Tetracycline (Aldrich/ Sigma) (5 mg/mL), Chloramphenicol (Aldrich/ Sigma) (34 mg/mL).

Agar Dilution

The anti-bacterial agents was measured by using broad spectrum antibiotics (ampicillin, tetracycline and chloramphenicol) of different concentrations (0.5, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/mL) and inoculated with standard tested organisms (*E. coli*, *S. aureus* and *P. aeruginosa*) which were prepared by mixing part of the growth from each of 5 similar colonies in saline and incubated at 37 °C for 2 hours.

Turbidity of the suspension was adjusted to 0.5 McFarland standards (BioMerieux, France) spectrophotometric ally at 600 nm wavelength (1 × 106 cell/mL) [9-11].

Preparation of Susceptibility Strip Test

The test strip consists of micro tubes containing dehydrated (lyophilized) media tryptic soya broth, glucose (5%) and bromothymol blue (0.1%) and broad-spectrum antibiotics in critical concentration tested for all antibiotics were equivalent to the MIC break point for susceptibility in sterile condition. The pH was adjusted to 7.4 (Alkaline, blue color).

Application of Strip Test

Bacterial suspension of particular microorganisms (*E. coli*, *S. aureus* and *P. aeruginosa*) were inoculated in tubes containing various antimicrobial agents. After incubation for 2 hours at 37 °C, results were reported (as a change in color of the media) by naked eye.

Conventional Antibiotic discs Method

Inoculums of the test organism were prepared as before. Sterile cotton swabs were depended in the test and control organisms separately. These swabs were used in inoculation of the specified areas of the Petri-dishes with test and control organisms.

Later flamed forceps used to apply Antibiotic discs with light pressure on the agar surface after the inoculums had dried. Finally, the Petri-dishes were incubated for 18-24 hours at 37 °C and the results were reported (radial width of the zones outside the antibiotic discs) by naked eye [11].

Results and Discussion

Table 1 show different concentrations of ampicillin tested against standard bacterial suspension of *E. coli*, *S. aureus* and *P. aeruginosa* were inoculated in the media. Tested critical concentrations in sterile condition for ampicillin were equivalent to MIC break point which were 4, 32, 256 µg/mL for tested bacteria, respectively. While, the average number of bacteria were 15.6×10^6, 12.9×10^6 and 2.1×10^6 bacteria/mL, respectively. The *E. coli* was more sensitive to ampicillin followed by *S. aureus* and then *P. aeruginosa*.

Table 2 shows the results of testing the sensitivity of standard pathological bacteria (*E. coli*, *S. aureus* and *P. aeruginosa*) for tetracycline using different concentrations of the antibiotics. Where it was noted that the focus MIC to the bacteria were 512, 512, and 32 µg/mL, respectively, and the average number of bacteria were 16.2×10^6, 16.2×10^6, and 5.4×10^6 bacteria per mL, respectively.

P. aeruginosa was seen to be more sensitive to tetracycline, whereas the bacteria *S. aureus* and *E. coli* had the same degree of sensitivity.

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Table 1 MICs of ampicillin for different concentrations *of E. coli*, *S. aureus* and *P. aeruginosa*.

Isolates	No. of bacteria(1 × 10₆) for different concentration of ampicillin (µg/mL)									
	0.5	2	4	8	16	32	64	128	256	512
E. coli	34.2	33.3	15.6	32.7	29.4	6.9	26.7	25.2	24	21
S. aureus	24.3	16.8	29.4	16.2	15.9	12.9	3.3	18.9	15	21
P. aeruginosa	20.4	8.1	7.8	21.9	8.4	4.9	3.3	2.4	2.1	1.8

Table 2 MICs of tetracycline for different concentrations of *E. coli*, *S. aureus* and *P. aeruginosa*.

Isolates	No. of bacteria(1 × 10₆) for different concentration of tetracycline (µg/mL)									
	0.5	2	4	8	16	32	64	128	256	512
E. coli	48.3	39.9	37.5	35.1	32.4	29.1	20.1	14.4	19.2	16.2
S. aureus	20.4	17.7	22.2	33.3	9.6	25.8	20.1	18.6	17.7	16.2
P. aeruginosa	13.5	10.5	7.2	21	5.1	5.4	7.2	8.7	7.2	6.6

Table 3 MICs of chloramphenicol for different concentrations of *E. coli*, *S. aureus* and *P. aeruginosa*.

Isolates	No. of bacteria (1 × 10₆) for different concentration of chloramphenicol (µg/mL)									
	0.5	2	4	8	16	32	64	128	256	512
E. coli	21.9	6.6	3.3	9.9	17.1	14.4	13.2	12.3	6	4.2
S. aureus	42.3	37.8	25.8	23.7	22.8	21.9	26.4	26.1	24.3	13.8
P. aeruginosa	15.6	11.1	6.6	29.1	7.5	21.9	10.2	3.9	5.1	4.8

Table 4 MICs and MBCs of ampicillin, tetracycline and chloramphenicol among *E. coli*, *S. aureus* and *P. aeruginosa*.

Isolates	Ampicillin		Tetracycline		Chloramphenicol	
	MIC, µg/mL	MBC, µg/mL	MIC, µg/mL	MBC, µg/mL	MIC, µg/mL	MBC, µg/mL
E. coli	4	32	512	128	512	4
S. aureus	32	64	512	16	32	512
P. aeruginosa	256	512	32	16	512	128

Whereas, Table 3 showed sensitivity test of different concentrations of chloramphenicol against the standard pathogenic strains (*E. coli*, *S. aureus* and *P. aeruginosa*). The MIC of the bacteria were 512, 32, and 512 µg/mL, respectively. The average number of bacteria were 4.2×10^6, 21.9×10^6 and 4.8×10^6 bacteria per ml, respectively. It was note that *S. aureus* was more sensitive to chloramphenicol, whereas the bacteria *E. coli* and *Pseudomonas aeruginosa* had the same degree of sensitivity.

In addition to the MIC value, the MBC (minimum bactericidal concentration) value of antibiotics was estimated for all pathogenic strains as it is shown in Table 4.

Though the values of the MBC of ampicillin were 32, 64, and 512 µg/mL for *E. coli*, *S. aureus* and *P. aeruginosa*, respectively, but MBC of tetracycline were 128, 16 and 16 µg/mL for standard bacteria, respectively.

The MBC of chloramphenicol were 4, 512, and 128 µg/mL, respectively for tested bacteria.

In this study glucose sugar selected in the preparation of the new test strip because most types of bacteria containing the enzyme fermented glucose sugar. Bromothymol blue dye was the most suitable dye used to indicate fermentation process and can note the color change clearly with the naked eyes as it is in Fig. 1.

The process of freeze drying had important role in maintaining culture media in the pockets of test strips and control small quantities used in addition to the ability to keep for a long time at low temperatures (4-6 °C).

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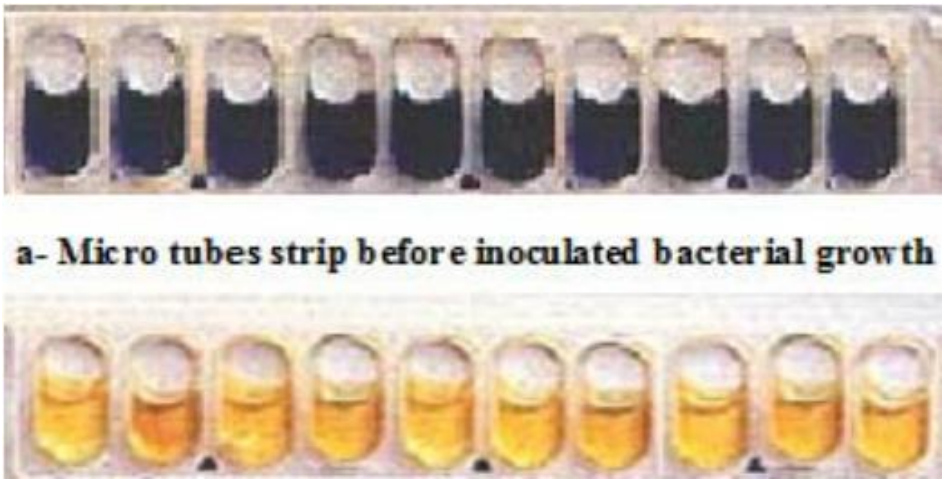
We concluded from the results of the study provide a great opportunity to work in diagnostic laboratories, wherever they are located inside or outside the provinces. The implementation of potential sensitivity



a- Micro tubes strip before inoculated bacterial growth

b- Micro tubes strip after inoculated bacterial growth

Fig. 1 Antibiotic susceptibility test performing micro tubes strip before (a) and after (b) inoculated bacterial growth.

Blue color : sensitive; Yellow: resistance bacteria.

testing and providing of best services, as well as reducing the cost and materials, all these required for economy support to achieve sensitivity test for bacterial types. Recognizable to tape record also reduced the period required to achieve the desired goal of 24 hours to 2 hours and this reduces the effort and increases the speed of the delivery of the required treatment to the patient. Finally, we recommend the Ministry of Health for the adoption of the way to ensure the test required in all diagnostic laboratories.

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تطوير طريقة جديدة لفحص حساسية البكتريا المرضية لبعض للمضادات الحياتية

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الخلاصة

طورت طريقة جديدة لفحص حساسية البكتريا للمضادات الحياتية في المختبرات الطبية وذلك باستخدام شريط الفحص (المكون من أنابيب أو جيوب صغيرة لاصقة بالشريط) يحتوي في داخله على الوسط الزرعي المجفد المتكون من مرق صويا التربتون، **5%** كلوكوز و**0.1%** صبغة البروموثايمول الزرقاء وأحد المضادات حياتية **Ampicillin, Tetracycline, Chloramphenicol)** وبتراكيز نموذجية (التركيز الأدنى المثبطة للنمو **MIC,**). استخدمت عالق العزلات البكتيرية القياسية (***Staphylococcus aureus, Escherichia coli*** و ***Pseudomonas* aeruginosa**) في الاختبار وتلقيح الوسط ، بعد أن ضبطت عكرة العالق مع عكرة محلول ثابت العكرة القياسي مكفرلاند **0.5 (1** × **10^6** وحدة تكوين المستعمرات / لمل الواحد (**CFU/ ml**) وحضنت لفترة ساعتين في**^oC 37** .

حددت التركيز الأدنى (**MIC**) المثبط للمضاد الحياتي **Ampicillin** لأنواع البكتريا (***Pseudomonas aeruginosa,* 4، 32، 256** مايكروغرام لكل مليليتر ولأنواع البكتريا على توالي. وكانت التركيز الأدنى للمضاد الحياتي **Tetracycline** لأنواع البكتريا **512، 512و32** مايكروغرام لكل مليليتر على التوالي. في حين كان التركيز الأدنى للمضاد**Chloramphenicol 512 ، 32 و 512** مايكرو غرام لكل مليليتر على التوالي.

كما لوحظت إن العزلات البكتيرية المقاومة للمضادات الحياتية تمكنت من النمو وتخمير سكر الكلوكوز والذي سببت في تغير لون الوسط من اللون الأزرق إلى الأصفر نتيجة تغير الـ**pH** ، بينما البكتريا الحساسة لم تنمو ، لذا لم تحدث تغير في اللون. قورنت نتائج البحث مع طريقة أقراص المضادات الشائعة استخداما وكانت النتيجة مطابقة.

تميزت هذه الطريقة الجديدة كونها سريعة (ساعتين فقط) وذات كلفه قليله جدا مقارنة بالتقنيات التقليدية. كما يميز شريط الفحص المبتكر بالاستقرار العالية (أكثر من سنه واحده) وحساسيتها في التشخيص العديد من البكتريا المرضية ضمنا العزلات البكتيرية القياسية مقارنة بالطرائق التقليدية.

الكلمات المفتاحية : الحساسية، MIC**، المضادات، تقنيات المختبرات الطبية**