Approach for screening of plasmid curing agents from microorganisms by agar overlay technique

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Abstract

Till date many compounds having an ability to cure the organisms of the bacterial resistance, have been identified. These compounds come from different sources like plants, oils (natural) and chemicals (synthetic). However, there are no reports of any plasmid curing compounds being identified from microbial sources. In this work we have tried to find whether microbes produce such compounds or not. We screened micro-organisms from soil We designed a novel technique of agar overlay for this purpose. This technique is relatively simple and efficient as compared other time consuming and laborious techniques used for screening. During this screening, potential microorganisms producing curing agents were found. Further work needs to be carried out to identify and characterize such natural compounds. These compounds could hold a promise for being used as potential additives to antimicrobials so as to make antimicrobial chemotherapy more effective.

Key words: plasmid curing agents , agar overlay technique , *E coli* , fungi

Approach for screening of plasmid curing agents from micro-organisms by agar overlay technique

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طريقة للتحري عن العوامل العلاجية البلازميدية من الاحياء المجهرية بواسطة تقنية تراكب الوسط

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الخلاصة

حتى الأن تم التعرف على العديد من المركبات التي لها القدرة على شفاء الكائنات الحية من مقاومة الجراثيم. هده المركبات تأتي من مصادر مختلفة مثل النباتات، والزيوت (الطبيعية) والمواد الكيميائية (الاصطناعية). ومع ذلك، لا توجد تقارير بان هذه المركبات او العوامل تنتج من البلازميد ذا مصادر جرثومية. في هذا العمل حاولنا أن نجد ما إذا كانت الميكروبات تنتج هذه المركبات من عدمه. تم التحري عن الكائنات الحية الدقيقة من التربة وتصميم تقنية جديدة من تراكب أجار لهذا الغرض. هذه التقنية تعتبر بسيطة نسبية وكفاءة مقارنة بتقنيات أخرى ذا وقت أطول وشاقة. خلال هذا التحري، تم العثور على الكائنات الحية الدقيقة المنتجة لتلك المواد. مزيد من العمل يتعين القيام بها لتحديد وتوصيف المركبات الطبيعية من هذا القبيل. لمكن لهذه المركبات أن تستخدم كمواد مضافة محتملة لمضادات الجراثيم وذلك لجعل العلاج الكيميائي المضاد للميكروبات أكثر فعالية.

Introduction

A plasmid is an extra-chromosomal DNA molecule separate from chromosomal DNA and capable of autonomous replication. It is typically circular and double stranded. It confers a number of useful properties to the host like:• Resistance to anti-microbial agents like antibiotics, synthetic chemotherapeutic agents, heavy metals , pigment production., toxin production. and , antibiotic production. (1)

The plasmids that carry genes conferring on the host cell resistance to antibiotics are called as ‘R factors.’ R factors are encountered in certain strains of almost all pathogenic bacteria. These R factors can be transferred to other bacteria in the environment and these extra chromosomal DNA sequences are responsible for the emergence of multiple drug resistant (MDR) strains. (2)

The over prescription and misuse of traditional antibiotics has led to the widespread antibiotic resistance in human pathogenic bacteria which justifies research on new possible antiinfective agents or resistance modifiers. An effective resistance modifier would be an anti plasmid compound which would eliminate (Cure) the plasmid from its host. Elimination of plasmids from their hosts would: make resistant bacteria again sensitive to traditional antibiotics. (3)

Despite their popular use the physical and chemical methods suffer from certain drawbacks. They are: general curing effect resulting in some or all plasmids being eliminated during the process of curing the virulence., possibility of mutational changes induced in the host during the curing treatment resulting in rough strains, reduction virulence due to unknown mutations other than loss of virulence plasmid. and Some of the plant extracts have been shown to induce allergies, toxic reactions or mutagenic effects. (4)

Goals of research

the agar overlay technique is relatively simple technique having many advantages. Firstly, it could be used to screen a large number of isolates from different sources like soil samples at a time. Secondly, a large number of natural curing compounds and antibiotics could be screened for by this technique.

Material and Method

Media composition:

* Rich Rawan (RR) broth (100ml)

Sodium acetate 0.5g ---Sodium citrate 0.5g ---Glucose 0.5g ---Peptone 0.5g ----Yeast extract 0.5g ---Sodium citrate 0.2g ----Distilled water 100ml

*Poor Rawan (PR) broth was prepared using 1ml of RR broth in 99 ml of water. For preparation of PR Agar, 2% Agar is added to the broth. Sterilized at 15 pounds per square inch.

Antibiotic Agar was prepared by adding antibiotic to make the final concentration 100µ/ml to Nutrient Agar. Here ampicillin stock of 50 mg/ml was prepared and was added as 0.2 ml per 99.8 ml medium. 29

All the media were sterilized at 15 pounds per square inch for 15 minutes

Methods:

1) Soil Samples: Soil samples used for isolating potential producers of antimicrobial agents (Reduction agents) were obtained from:· Garden soil from Baghdad University - college of science -department .of Biology

2) Bacterial isolates used as stimulated organisms (to Fungi and other microorganisms soil samples to produce antimicrobial agents to fight back this strains) was *Escherichia coli* (ampicillin resistant) (5)

3) Culture media used:

- For Screening: Poor Rawan agar media and ampicillin agar was used.
- For preparation of cell free broth: Rich Rawan, Poor Rawan,.(6)

4) Agar overlay method used for screening from organisms from soil.

Soil samples obtained are homogenized and large particles from them are removed. and 0.5 g of the homogenized soil sample is weighed on an electronic balance. then this soil sample is serially diluted (10 fold dilution) in sterile saline up to 10^{-6} dilution. and subsequently ☒dilutions 10^{-4}, 10^{-5} and 10^{-6} are platted out by pour platting technique in sterile Poor Rawan (PR) medium after that plated are incubated at 37^{0}C for 1 week to obtain well isolated colonies. then plates are overlaid with sterile antibiotic agar. and the plasmid bearing test organism (24 hour old culture) is spread over the hardened upper layer so as to get matt growth finally plates are then kept for pre-diffusion in the refrigerator for 48 hours and . the plates are incubated at

37 C for 24 hours , after incubation, plates are observed for zones of inhibition of test organism.(7,8)

5) Differentiating between curing and antibiotic activity. The inhibition of the plasmid bearing test organism observed could be due to 2 reasons:

. Due to another antibiotic produced by the isolate to which the test organism is sensitive .or due to the production of a plasmid curing agent by the isolate. For distinguishing between these 2 possibilities the further experiment was performed .thereby the suspected isolate is streaked in the form of a thick band on 2PR plates then one plate was overlaid with Nutrient agar and the other plate was over laid with Antibiotic agar., after that plasmid bearing test organism (24 hour old culture) was cross streaked on both plates and plates are kept for prediffusion in the refrigerator for 48 hours and incubated at 37 C for 24 hours finally the plates are observed for zones of inhibition around the streaks of the plasmid bearing test organism.(9)

6) Checking activity in cell free broth using well diffusion method.

Well diffusion assay for checking activity in cell free extract.and sterile Antibiotic Agar plates are prepared and test organism is spread onto these plates. then well are punched using a flame sterilized borer. After that cell free extracts are loaded into the wells and suitable controls were maintained., finally Plates are kept for pre-diffusion in the refrigerator for 24 hours .and incubated at 37 C for 24 hours. (10 ,11)

Result

I] Preliminary Screening:

The isolates from soil were obtained by the agar overlay method which could significantly reduce the plasmid bearing *E coli* strains

First isolate resist to Amp. antibiotic (carried Amp plasmid from transformation technique)

Second isolate resist to Clox.. antibiotic (carried Clox. plasmid from transformation technique)

Third isolate resist to Amp ,Clox. and Gen.. antibiotics (carried Amp ,Clox. and Gen plasmids from transformation technique)

Fourth isolate resist to Amp and Gen. antibiotics (carried Amp. and Gen plasmids from conjugation technique)

Fifth isolate resist to Amp and Clox. .antibiotics (carried Amp. and Clox. plasmids from conjugation technique)

Sixth isolate resist to Amp ,Clox. and Gen.. antibiotics (carried Amp ,Clox. and Gen plasmids from conjugation technique)

II] Curing activity of the isolates:

Now the inhibition of the plasmid bearing test organism could be due to production of a new antibiotic by the isolate or due to production of a plasmid curing agent .To differentiate between these two possibilities, the isolates were band streaked on 2 different plates and were overlaid with either Nutrient agar or Antibiotic agar. The plasmid bearing test organism was cross-streaked across the hardened upper layer. Results were interpreted as follows:

Table (1) determining the production of different isolates to curing agent and antibiotics

Overlay		Possibility
Antibiotic Agar	Nutrient agar	
significantly reduction	No significantly reduction	the isolate is producing a curing agent.
significantly reduction	significantly reduction	the isolate is producing curing agent and antibiotics
No significantly reduction	No significantly reduction	The isolate is neither producing an antibiotic nor a plasmid curing agent.

Table (2) determining the result of different isolates to curing agent and antibiotics

Overlay		Isolate
Ampicillin and Clox.and Gen.Agar	Nutrient Agar	
-	-	First isolate
-	-	Second isolate
+	-	Third isolate
+	-	Fourth isolate
+	-	Fifth isolate
+	+	Sixth isolate

+: significantly reduction ; - No significantly reduction

From the result above we can predict that the first isolatewhich is plasmid bearing Amp. resistant showed no significantly reduction for both antibiotics agar and nutrient agar .

The second isolatewhich is plasmid bearing Clox. resistant showed no significantly reduction for both antibiotics agar and nutrient agar .

The third isolatewhich is plasmid bearing Amp. and Clox. resistant and Gen resistant carried on chromosome showed significantly reduction in antibiotics agar meanwhile it showed no significantly reduction in nutrient agar

The fourth isolate. which is plasmid bearing Amp. resistant and Gen resistant carried on chromosome showed significantly reduction in antibiotics agar meanwhile it showed no significantly reduction in nutrient agar

The fifth isolate. which is plasmid bearing Amp and Clox.. resistant showed significantly reduction in antibiotics agar meanwhile it showed no significantly reduction in nutrient agar

The sixth isolatewhich is plasmid bearing Amp. and Clox. resistant and Gen resistant carried on chromosome showed significantly reduction for both antibiotics agar and nutrient agar

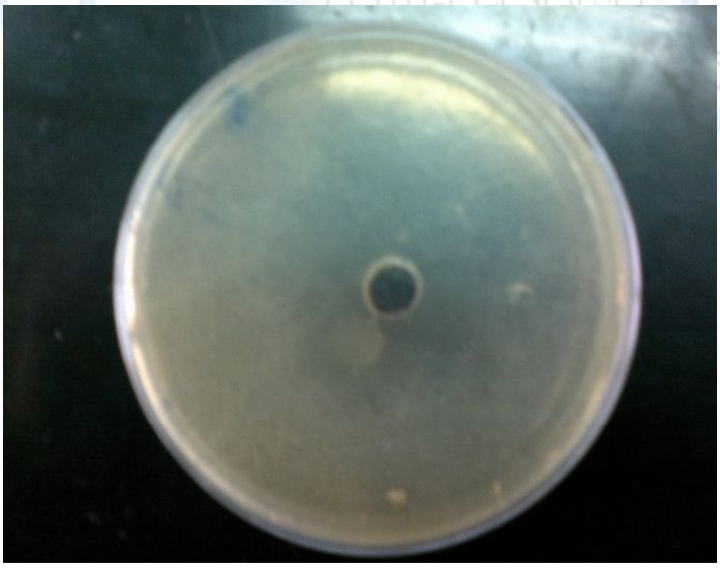
Checking activity in the cell free broth:

+: Inhibition; - No Inhibition

All isolateshowed no inhibition for cell free extract checking activity only third and fifth which showed the inhibition zone.

Table (3) Checking activity in the cell free broth for different isolates

	Isolate	Results
	First strain	-
	Second strain	-
	Third strain	+
	Fourth strain	-
	Fifth strain	+
	Sixth strain	-



(1) inhibition of third isolateresist to Amp ,Clox. and Gen.. antibiotics (produced by transformation technique) by cell free extract

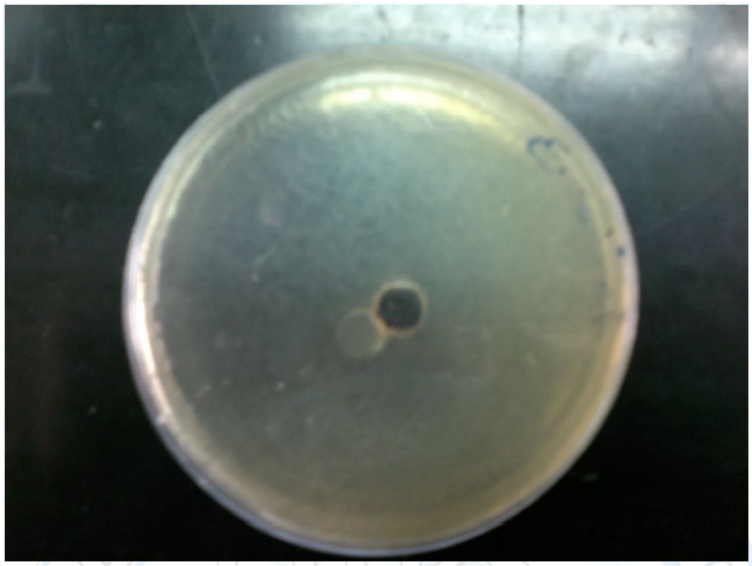


Figure (2) inhibition of Fifth isolateresist to Amp and Clox.. antibiotics (produced by conjugation technique) by cell free extract

Discussion

While checking for the curing activity in cell free extracts, This activity is effected by many factors that include:

- Number of plasmid in bacterial strains under the effect of curing compounds
- The plasmids of different bacterial strains came form conjugation or transformation process

The (third isolate) which is plasmid bearing Amp. and Clox. resistant and Gen resistant carried on chromosome , (fourth isolate) which is plasmid bearing Amp. resistant and Gen resistant carried on chromosome and (fifth isolate). which is plasmid bearing Amp and Clox.. resistant showed significantly reduction in antibiotics agar meanwhile it showed no significantly reduction in nutrient agar then the isolate is producing a curing agent for plasmid resist (Amp. and Clox.)

While the cell free extract contained curing agent for plasmid resist to the (Amp. and Clox.) ,(first isolate) which is plasmid bearing Amp. resistant and (second isolate) which is plasmid bearing Clox. resistant showed no significantly reduction for both antibiotics agar and nutrient agar .the reason for that might be the activity of curing agent effected by number of plasmid in bacterial strains .

First and second isolatecontained only one plasmid while third and fifth contained two plasmid and the cell can keep one plasmid .more stronger than keeping more than one, therefore curing agent can effect on two plasmid more severely than effect on one plasmid.

While fourth isolatecontained only one plasmid but the curing agent of cell free extract effected on bacterial cell , this is because the fourth isolatecarried plasmid resist to Amp. (Produced by conjugation process) while the first and second isolate(produced by transformation process) and the stability of plasmid in transformation more than conjugation process .

The sixth isolatewhich is plasmid bearing Amp. and Clox. resistant and Gen resistant carried on chromosome showed significantly reduction for both antibiotics agar and nutrient agar

Thus, the agar overlay technique could be a reliable technique for screening for different products like antibiotics, plasmid curing agents from micro-organisms. Thus, the race between antibiotic producers and resistance developers can be exploited to obtain large number of such compounds. Thus, the microbial world can be a potential area for existence of natural plasmid curing compounds.

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