EXTRACTION AND PURIFICATION OF COLICIN PRODUCED BY*ESCHERICHIA COLI* ISOLATED FROM URINARY TRACT INFECTION[+]

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Abstract

1017 urine samples were cultured from patients suffering from urinary tract infection. Out of these 648 isolates were obtained and 369 gave no bacterial growth . *E. coli* showed the high percentage 42.1% followed by *Proteus mirabilis* 12% ,*Klebsiella pnuemoniae* 8.7%, *Psuedomonas aeruginosa* 7%, and *Enterobacter spp* 6.3% . While the percentage of Gram positive bacteria (*Staphylococcus aureus, Streptococcus pnuemoniae* and *Staphylococcus saprophyticus* were 10,1%,8.3%and 5% respectively.

The local *E. coli* isolates from UTI were examined for colicin production by exposing each isolate to the rest of all others and looking for the inhibition of the growth, three methods for this test were employed , cup assay , wells assay and cross streaking , Cup assay was found to be the most efficient , thus was used throughout the study.

Six types of modified media were used to choose the best one (give highest production of colicin , brain heart with 5% glycerol was the most efficient medium for colicin production . colicin was extraction by homogenization with NaCl which gave an activity 640 U\ ml.

Purification of crude colicin had been carried out by using ammonium sulphate result indicated that (40-60) was regarded as the best saturation ratio for colicin precipitation , the specific activity of colicin after the dialysis was 1066.6. The activity spectrum of purified colicin was studied and found to affect the isolates which caused UTI.

المستخلص

زرعت الف وسبعة عشر عينة إدرار من الأشخاص الذين يعانون من التهاب المجاري البولية ووجد إن ٦٤٨ عينة كانت موجبة الزرع المختبري شكلت نسبة ٧٢,٥% اما ٣٦٩ عزله كانت سالبة الزرع المختبري . كذلك وجد إن بكتريا اشيريكيا القولون هي المسبب الرئيسي لالتهاب المجاري البولية إذ شكلت نسبة ٤٢,١ % من مجموع الإصابات الكلية تلتها بكتريا بروتيس ميرابيلس ١٢% ، والكلبسيلا الرئوية ٨,٧% والزوائف الزنجارية ٧% ثم بكتريا الانتيروبكتر بنسبة ٦,٣% بينما كانت نسب الإصابة بالبكتريا الموجبة لصبغة كرام متمثلة ببكتريا المكورات العنقودية الذهبية والمسبحيات الرئوية والمكورات العنقودية الرمية هي ١٠,١ % ،

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٨,٣%، ٥% على التوالي . اختبرت قدرة عزلات اشريكيا القولون المحلية على إنتاج الكوليسين ووجد من خلال هذه الدراسة إن ٤٨ عزلة من هذه العزلات كانت منتجة للكوليسين شكلت نسبة ١٧,٥% وقد استخدم لهذا الغرض ثلاث طرائق هي

Cup assay , Wells assay and Cross streaking assay

واتضح من خلال هذه الدراسة ان طريقة Cup assayهي الطريقة المناسبة لغرض التحري عن الكوليسين كذلك استخدم في هذه الدراسة ستة أنواع من الأوساط الزرعية للتحري عن الكوليسين ووجد إن وسط نقبع القلب والدماغ مضافا إليه ٥% من الكليسيرول هو الأفضل للتحري عن النشاط الكوليسيني لبكتريا اشيريكيا القولون

تم استخلاص الكوليسين بوساطة NaCl ، ووجد إن فعاليته كانت ٦٤٠ وحدة / مل ، واستخدمت طريقة الترسيب بأملاح الامونيوم لغرض التنقية وبنسبة اشباع (٤٠-٦٠)% وكانت هي النسبة المثلى لغرض الترسيب إذ وجد إن فعالية الكوليسين النوعية لاسيما بعد عملية الديلزة هي ١٠٦٦,٦ . كذلك وجد إن للكوليسين تأثيرا على البكتيريا المرضية المعزولة من التهاب المجاري البولية

Introduction

Urinary tract infection (UTI) is the most common of all infections and can occur at any time during the life of an individual . Almost 95% of cases of UTIs are caused by bacteria. The importance of UTI has been recently increased since it remain the most common among total acquired infections [1,2]

One of the most important bacterial pathogen is *Escherichia coli* (E. coli) which is also the etiology of many diseases in man and animals [3 ,4]. The importance of *E. col*i was increased since it produces variety of toxins and other virulent factors which plays a significant role in its pathogenesis and increase its ability to colonize and cause disease[5,6] .

One of the most important factors affecting its prevalence is the production of colicin [7, 8]. The production of colicins is a common trait in *E. coli* [9] . The term colicin encompasses a diverse class of antimicrobial agents, with over twenty types of feature [10 , 11] .The aim of this study is to detect the colicins produced by local *E. coli* isolates from patients with UTI by three methods using different types of media in order to purify this colicin.

Materials and Methods:

*** Source of E. coli isolates**:

Mid-stream, urine samples (1017) were cultured from patients suffering from UTI. Isolates were identified according to [1]. 273 isolates belong to E. coli .

*** Detection of colicinogenic isolates**:

This was performed by exposing each of the 273 *E. coli* isolates to the rest of all others and looking for the inhibition of the growth .For this purpose, Three methods were used to detect the production of colicin:

Cross streaking method [12] ; Wells Assay method [13] ; Cup Assay method [14].

***Selection of Isolate for assay**:

The higher producer isolate out of the *E. coli* isolates which was chosen to study its colicin (c), this isolate chosen to study its colicin effect on several pathogenic bacteria caused UTI and conditions production despite using different type of media.

***Culture media:**

After isolating bacteria on MacConckeys and blood agar at 37^{o} C for over night incubation. The indicator isolates were cultured on nutrient broth and nutrient agar while colicinogenic isolates were cultured on brain heart infusion broth, nutrient agar , brain heart infusion agar, nutrient agar with 5% glycerol, brain heat infusion agar with 5% glycerol and Luria broth medium.

Luria broth medium and brain heart infusion broth were used to determine the activity of killing of colicin in supernatant .

***Colicin Activity Assay:**

The quantitative determination of colicin in bacterial culture was performed by using the Wells Assay [15].and direct spotting method [16].

Serially diluting 10% v\v chloroform treated culture in nutrient broth onto an agar plate freshly seeded with 10^{7} bacteria/ ml indicator bacteria and cell free preparation of colicin were assayed for activity.

Protein determination was done according to[17] and the Mitomycin-C was used as an inducer for colicin production in luria broth medium which contained the producer isolate.

***Specific Activity:**

The specific activity is defined as the number of colicin units per mg protein.

***Growth and Induction of *E. coli* (C) for colicin Production:**

Twenty five ml of an overnight culture of *E. coli* isolate (C) in Luria broth medium was used to inoculate. 1000 ml sterile Luria broth and incubated in shaking water bath at 37^{o} C.The cell density of about 3 x 10^{8} cell / ml after 14 th hr.incubation. Mitomycin-C was added to concentration of 2 mg/ml ,incubation continued with shaking for 5 hrs. A sample of the culture of cell as sample of supernatant and of the cell pellet (after centrifugation) were made for assay of colicin activity and protein determination.

*** Salt Extraction of colicin:**

According to[18],extraction of colicin from the packed cell was carried out by addition of 30 ml of 1.0 M NaCl in standard potassium phosphate buffer (PPB) in manual homogenizer in three successive washes, homogenization was carried out according to [19]. After extraction the cell suspension was centrifuged for 10 min. at 5000xg, and the supernatant was sucked and saved. Extraction of cell pellet was repeated 3 times and the 3 extracts were pooled and referred to as
(Crude colicin preparation).For each step, colicin activity ,protein concentration and specific activity were determined .[19]

***Purification of colicin:**

According to [20] , the volume of pooled colicin extract was adjusted to 50 ml with NaCl extraction buffer. Solid ammonium sulphate was slowly added to crude colicin extract with continuous stirring for 30 min. to precipitate the low molecular weight proteins at 40% solid ammonium sulphate was further added to the supernatant to bring the concentration up to 60% with continuous stirring in ice bath for 30 min. Suspension then centrifuged at 8000Xg for 30 min

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The supernatant was discarded and precipitate containing colicin was resuspended in 15 ml standard PPB . Following resuspension it was centrifuged again at 8000xg for 30 min. Then the clear colicin was dialyzed for 6 hr. Against 4 liters standard PPB changed 3 times. Dialysis against sucrose for 12 hr.was done to concentrate the volume to 2 ml for each step ,colicin activity , protein concentration and specific activity was determined .

***Activity Spectrum of Purified Colicin:**

Effect of purified colicin on pathogenic bacteria was determined as follows:

0.1 ml from broth culture of pathogenic bacteria was spreaded on nutrient agar surface after 15 min. serial dilution from purified colicin were added. Purified colicin observe their effect on pathogenic bacteria and detect by using patch test [21].

Results and Discussion:

Bacterial Isolates:

One thousand-seventeen urine samples of UTI were collected and cultured on MacConkeys and blood agar. Out of which 648 samples
(63.7 %) showed growth of bacteria and the rest 369(36.3 %) no growth of any bacteria was detected. Bacterial isolates were identified according to morphology of colonies and cells under microscope after staining with gram stain, then identification was confirmed by biochemical tests.

The isolates identified were *E. coli ,Proteus mirabilis ,Klebsiella pnuemoniae, Psuedomonas aeruginosa , Enterobacter spp , Staphylococcus aureus, Streptococcus pnuemoniae and Staphylococcus saprophyticus*. The percentage of each are summarized in table (1). From this results the common cause of UTI was *E. coli* 273(42.1%) . *E. coli* is the major pathogen in upper and lower UTI in human [22 ,23] . So it has been chosen to be studied due to its high incidence as common cause of UTI .This result was in agreement with other studies [24 ,25].

Detection of Colicinogenic E. coli Isolates:

Colicin is one of the important virulence factors which helps this genus to colonize and help other bacteria to cause infection , and for detection of colicinogeny, cup assay was used Table (2). shows that out of 273 *E. coli* isolates 48 (17.5 %) were colicin producer isolates and 225(82.5%) are not producer.

Table (1): Bacterial spp isolated from UTI

Type of isolates	No. of isolates	Percentage
E. coli	**273**	**42.1**
Proteus mirabilis	**78**	**12**
Klebsiella pnuemoniae	**57**	**8.7**
Psuedomonas aeruginosa	**46**	**7**

Enterobacter spp	**41**	**6.3**
Staphylococcus aureus	**66**	**10.1**
Streptococcus pnuemoniae	**54**	**8.3**
Staphylococcus saprophyticus	**33**	**5**
total	**648**	**100**

Table (2) : Detection of colicin production in 273 E. coli isolates by cup assay method.

Total positive urine sample	No. of E. coli isolates		No. of colicinogenic E. coli isolates	
	No.	%	No.	%
648	273	42.1	48	17.5

Approximately 30- 40 % of pathogenic *E. coli* from different serotypes were found to synthesize colicin [26 ,27]. The production of colicin inside the gut or urinary tract may play a role in changing the environment of these system, although producer and sensitive cells have been isolated from the same environment [28] .Our result disagree with other studies such as [29 ,30], because they mentioned high percentages 88% , 33% respectively. Also the amount of the colicin produced plays an important role,i.e. the large amount the highest effect, because killing of sensitive cells depends on concentration of colicin molecules [31].Table (3) demonstrates percentage of sensitive isolates which are inhibited by the producer isolate(C) .Table (3)show that the appropriate method for detection of colicin production was the cup assay method which showed a number of sensitive isolates higher than the other methods (range between 63-142) sensitive isolates. The best medium used was brain heart infusion with 5% glycerol because the number of sensitive isolates was higher than that on other types of media, the range (75- 142) sensitive isolates. This result is in agreement with other studies [32 ,33]. The cup assay is an efficient method for detecting colicin in local *E. coli* isolates and brain heart infusion supplemented by 5% glycerol is the best media to be used for enhancement of colicin production

Table (3):Relationship between methods of colicin detection in 273 local E. coli isolates and types of media .

Producer Isolate (c)	Types of Media					
	N. media	N. media +5%gly.	B.H.I media	B.H.I +5% gly.	L. media	L.media +5% gly.
	No. of sensetive isolates	No. of sensitive isolates	No. of sensitive isolates	No. of sensitive isolates	No. of sensitive isolates	No. of sensitive isolates
Cup assay	**63**	**71**	**93**	**142**	**82**	**95**
Wells assay	**45**	**52**	**88**	**102**	**75**	**79**
Cross streaking	**59**	**68**	**74**	**75**	**43**	**81**

Table(4):Relationship between colicin activity, protein concentration ,specific activity and steps of colicin purification

Steps	Activity U\ml	Protein concentration	Specific Activity

Supernatant	**320**	**16.3**	**19.6**
Crude colicin	**640**	**20.7**	**30.9**
Precipitation with Ammonium sulphate (40%-60%)	**640**	**3.4**	**188.2**
Dialysis	**1280**	**1.2**	**1066.6**

Extraction and purification of colicin:

The higher producer isolate ,out of 48 isolates which were chosen to study its colicin was (C) . The indicator isolates choose for assay of colicin activity throughout the study the best ones due to their sensitivity even by using different types of media in three different methods,
Table (3) . Spotting method was always used for estimation of colicin activity throughout the steps of extraction and purification because this method has the advantage of requiring only one cycle of incubation [34]

Mitomycin-C was used as an inducing agent and used in 2 µg /ml concentration . one of the most efficient ways of producing a large amount of colicin by inducing the bacteriocinogenic strain with suitable metabolic inhibitor, one of the most effective is Mitomycin –C [10 , 35].

Mitomycin –C is very suitable to use and increase the production of colicin 4 – 20 folds as compare to other inducer such as UV [36].

The concentration of 2µg\ml was more inducible for colicin production [32], therefore it was used throughout the study .

The colicin activity, protein concentration and specific activity for each step are measured and summarized in table (4).
Broth culture with Mitomycin –C increased colicin production on the other hand Nacl salt extraction was used instead of sonic extraction because [35] and [7] indicated that the colicin lost activity by using latter methods , NaCl salt extraction found to be together with homogenization leads to increase the specific activity.

Ammonium sulphate fractionation yields increase in specific activity . However protein concentration was decreased as step progressed . Also we could conclude that these extraction steps did not affect colicin activity.$(NH_4)SO4$ was used for precipitation which was also found to be very efficient and did not affect the activity shown by increasing the specific activity along together with proceeding steps table (4). [37].

Table (5) :*Activity spectrum of purified colicin on different types of bacterial isolates Which isolated from UTI.

Types of bacterial isolates	**No.of Isolates**	**No.of positive isolates**	**% 0f positive isolated**	**Mean diameter of inhibition zone**
E. col	**273**	**142**	**52.01**	**9.8**
Proteus mirabilis	**78**	**53**	**67.9**	**8.75**
Klebsiella pnuemoniae	**57**	**40**	**70.1**	**6.5**
Psuedomonas eruginosa	**46**	**22**	**47.8**	**7.6**

Enterobacter spp	**41**	**38**	**92.6**	**7.2**
Staphylococcus aureus	**66**	**28**	**42.4**	**5.3**
Streptococcus pnemoniae	**54**	**31**	**57.4**	**8.3**
Staphylococcus sarophyticus	**33**	**18**	**54.5**	**7.1**

* Patch test

Activity Spectrum of colicin :

The colicin from the local *E. coli* (C) isolated from UTI patients shows a wide activity spectrum for example it is effective among several genera and species of Gram negative and Gram positive bacteria table (5) . It has a wide range effect on *E. coli* 142 (52.01%); *Proteus mirabilis* 53 (67 .9%) ; *Klebsiella pnuemoniae* 40 (70.1%); *Psuedomonas aeruginosa* 22 (47.8%) ; *Enterobacter spp* 38(92.6%); *Staphylococcus aureus* 28(42.4%) ; *Streptococcus pnuemoniae* 31 (57.4%) and *Staphylococcus saprophyticus* 18 (54.5%).This is in agreement with [33 ,38 ,39]. The activity spectrum may help in the possibility of using colicin for used as epidemiological marker by studding typing of *E. coli* or other bacterial species according to their sensitivity to the colicin.

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