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Isolation, Identification and Partial Characterization of Plasmid DNA of Different Bacterial Species Isolated from Clinical Specimens of Patients Suffering from Diarrhea

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ABSTRACT

Out of 200 fecal specimens from patients admitted to the various hospitals in Erbil city suffering from acute and persistent diarrhea, it was found that the bacterial pathogens were recovered from 69 patients representing 34.5%. All bacterial isolates were identified by unique features of morphological, cultural, biochemical and serological characteristics. The enteropathogenic *Escherichia coli* (EPEC) was the most common group isolated from 33(16.5%) of the patients followed by *Salmonella*, *Klebsiella*, Enterobacter, *Proteus*, *Morganella* in ratios 13(6.5), 7(3.5), 6(3%), 2(1%), 2(1%) respectively. In addition, amplification of plasmid DNA content by antibiotics in some isolated bacterial species has been studied and the results showed enormous increase in plasmid DNA concentrations in amplified cultures of *Klebsiell*a *oxytoca, Salmonella* and *E. coli* isolates after treatment with 150 µg/ml chloramphenicol reaching to more than six folds of increase when compared with their concentrations in unamplified cultures. Also, in amplified cultures of other *K. oxytoca* and *E. coli* isolates but in presence of 150 µg/ ml tetracycline, the folds of plasmid DNA amplification appear to be in the range of 6.87 to 11.25.

عزل، تشخيص وتوصيف جزئي لمحتوى الـ DNA البلازميدي للأنواع الجرثوميه المعزولة من عينات طبية لمرضى يعانون من الإسهال

الملخص

من مجموع 200 عينة براز ماخوذة من مرضى مصابون بالاسهال الحاد والمزمن من مستشفيات مدينة اربيل وجد بان 69 حاله منها كانت بسبب عوامل جرثوميه والتي تمثل نسبة 34.5%. فحصت العزلات الجرثوميه مجهريا وشخصت باستخدام الاختبارات المزرعيه والمظهريه والمصليه والكيموحيويه وقد كانت جرثومة *E. coli* الممرضه للامعاء الاكثر شيوعا حيث عزلت من 33 مريضا وبنسبة 16.5% تبعها كلا من الاجناس الجرثوميه التاليه *Enterobacter, Klebsiella, Salmonella*, *Morganella, Proteus,* وبنسب 13(6.5)، 7(3.5)، 6(3%)، 2(1%)، 2(1%) على التوالي. اضف الى ذلك فقد تم دراسة قابلية تضخيم محتوى ال DNA البلازميدي بوساطة المضادات الحيويه في بعض

لانواع الجراثيم المعزوله واظهرت النتائج ان هناك زيادة كبيرة في تركيز ال DNA البلازميدي المعزول من المزارع المضخمه لعزلات ال *Salmonella* و *E. coli* بعد المعامله ب 150 مايكروغرام/ مل من الكلورامفينكول حيث وصل مقدار الزيادة الى اكثر من ستة اضعاف عندما تقارن بتراكيزها في المزارع غير المضخمة. كذلك في مزارع مضخمه اخرى من الانواع الجرثوميه *K. oxytoca* و *E. coli* ولكن بوجود 150 مايكروغرام / مل من التتراسايكلين فان مقدار التضخيم لل DNA البلازميدي يظهر انه ضمن مدى من 6.87 الى 11.2 ضعف.

INTRODUCTION

Diarrhea is the passing of increased amounts of loose stools. It is often caused by virus (mostly rotavirus), protozoa, (especially cryptosporidia, Giardia and amoeba) or bacteria (virtually most are Gram- negative bacteria) (Behrman et al., 2001).

Bacterial infections are very important causes of diarrhea in infants and children, particularly in developing countries and in other settings where standards of personal and community hygiene are low (Gracey, 1986). The enteropathogenic *E. coli* is an important cause of acute infantile diarrhea both sporadic and epidemic especially in developing countries and the disease is rare in adults. In Brazil and India, EPEC was largely responsible for diarrhea representing 25% and 34% in infants respectively (Vallance and Finlay, 2000). In contrast, the isolation rate of EPEC was low in Bahrin and Greece, estimated as 2% and 3.9% respectively, such low rates tend to be observed in countries with improving hygiene standards (Guerrant, 1983; Krishnamurthy, 1990). *Salmonella* species were the most frequently isolated organisms from stool specimens of diarrheal patients in the island of Crete and Greece (Samonis et al., 1997). Several other new bacterial agents have also been identified which would not have been detected in earlier studies of the epidemiology of infectious diarrhea, these include *Aeromonas*, *Camplybacter*, *Yersinia* and *Clostridium difficile* (Kain, 1993).

Recently, hundreds of different types of plasmids are found in members of enterobactericeae. There are at least 30 different incompatibility group and up to seven different types of plasmid have been found one in *E. coli* cell. Other than *E. coli*, plasmids are found in almost all members of enterobacteriaceae (Nicklin et al., 2001), and essential feature of bacterial plasmids are their ability to amplify their copy number. In bacteria, amplification occurs either spontaneously or induced and may aid the organism to adapt to particular environmental condition, e.g. a substrate normally used inefficiently by a given strain and may be used more efficiently following amplification of genes specifying the appropriate enzyme (Slingleton and Sainsbury, 1995).

The research involves isolation and identification of the bacterial species from specimens of patients suffering from diarrhea then detection the ability of these species to amplify their plasmid DNA by using antibiotics.

MATERIALS AND METHODS

Collection of specimens:

A total of 200 specimens of stool from infants, children and adults suffering from acute and persistent diarrhea were collected within four seasonal months (April to August 2005), from Hawler teaching and Maternity and Pediatrics hospitals in Erbil city. The

specimens were kept in clean sterile disposible plastic containers and delivered directly to the laboratory.

Isolation of etiological agents of diarrhea:

Specimens received for detection of the most frequently isolated Enterobacteriaceae were plated onto supportive enrichment media (blood agar and selenite broth) (Oxoid), a slightly selective and differiential media (MacConkey agar and Eosin–methylene blue agar (EMB)(Oxoid) and a moderiately selective media, Xylose-Lysine Deoxycholate (XLD)(Oxoid), Hektoen Enteric agar (HE), Salmonella-Shigella agar (BioMeriux) and Kligler iron agar (KIA)(Oxoid).

Biochemical Tests:

Api 20E system:

This system devised for identification of Enterobacteriaceae and other gram negative bacilli biochemically. It consists of 20 microtubes containing dehydrated media (each microtube consist of a tube and cupul section). The Api 20E system was performed according to the manufacture instructions.

Also Catalase and Cytochrome oxidase tests were carried out according to Cheesbrough (1992).

Serological tests:

The presence of Enteropathogenic *E. coli* was detected in the centeral laboratory of Al-Jumhuria hospital using specific antisera (Institute Pasteur Production). *E. coli* isolates were tested with polyvalent OK using slide agglutination method. Three drops of physiological normal saline added on a clean slide, then with aid of loop a small piece of fresh bacterial growth was mixed with the drops in order to obtain a homogenous mixure.

Afterthat a drop of polyvalent antisera was added to two drop (mixure) while the third drop considered as control test. The polyvalent antisera mixed gently with the bacterial mixure, appearance of clear agglutination within one minute indicate a positive result. No serological tests for identification the species of the *Salmonella* isolates were carried out because no kit available for this purpose.

Examining the ability of plasmid DNA to amplify its copy number in the isolated bacteria:

Amplification of the plasmid DNA content in the bacterial isolates was carried out in presence of the antibiotics chloramphenicol and tetracycline separately and according to the method of Norgard et al. (1979).

Plasmid DNA was extracted according to Birnboim and Doly (1979), from 50 ml amplified bacterial culture and unamplified one.

The purity of the prepared plasmid DNA was checked on nutrient agar plates for any contamination with bacterial cells occur. Then the optical density at 260 nm of the plasmid DNA yield was taken using UV-Spectrophotometer. Then, the DNA concentration calculated according to the method used by Ahmad (1989).

RESULTS AND DISCUSSION

Isolation and identification of the isolated bacteria:

cultural characteristic of the isolated bacteria:

All *E. coli* isolates characterized by producing small, smooth, entire and convex colonies on blood agar. They are red pink lactose fermenting on MacConkey agar and by producing bright metallic green sheen colonies on EMB agar.

All *Samonellae* isolates characterized by producing small, smooth, transparent, non- lactose fermenting colonies on MacConkey agar while producing red colonies with black centers on XLD agar. On HE agar *Salmonellae* isolates produce dark black colonies due to hydrogen sulphide production.

Enterobacter and *Klebsiella* isolates both produce large, viscous, mucoid colonies on blood agar while on MacConkey agar both produce large, viscous, light pink mucoid colonies due to lactose fermentation.

Morganella morganii produce small, convex, smooth, entire colonies on blood agar, it resemble colonies of *Proteus* species but no swarming type growth. On MacConkey agar it produce small pale transparent, non-lactose fermenting colonies while on XLD agar produce small individual red colonies.

Biochemical Reactions:

On Kligler Iron agar (KIA) medium the isolates showed the following phenotypic traits. *E. coli* isolates produce a yellow slant (acid production) and a yellow butt due to fermentation of lactose and glucose. All *Salmonellae* isolates produce a yellow butt and a red–pink slant (alkaline production) indicating fermention of glucose only and a black bottom referring to hydrogen sulphide production. *Enterobacter* and *Klebsiella* species produce a yellow slant and yellow butt indicating fermentation of lactose and glucose. *Morganella* produce a yellow butt and a red pink as a result of glucose fermentation.

Api 20E system:

After applying the Api 20E system to the different types of the bacterial isolates which primarily identified by cultural and biochemical characteristics, the Api test results revealed that there are. thirty nine isolates of *E. coli*, 13 isolates belonging to *Salmonella* species, 7 isolates of *Klebsiella* species (*K. oxytoca* and *K. pneumoniae*), 6 isolates of *Enterobacter* species (*E. cloacae*), 2 isolate of *M. morganii.*

In addition, all bacterial isolates understudy showed negative cytochrome oxidase and positive catalase tests.

Serological tests:

Results of the tested *E. coli* isolates with polyvalent OK using the slide agglutination technique revealed that 33 among 39 diarrhiagenic *E. coli* isolates revealed clear agglutination within one minute indicating positive results and considered as Enteropathogenic *E. coli.*

Bacterial prevalence and etiology of diarrhea:

The stool specimens of 200 patients suffering from diarrhea were examined and it is found that bacterial pathogens were recovered from the stool of 69 patients

representing 34.5% . From these 200 stool cultures, 67(97.4%) yielded a single enteric bacterial pathogen whereas 2(2.6%) of the isolates revealed multiple kinds of pathogens.

Diarrhiagenic *E. coli* was the most frequent bacterial isolates representing 39(19.5%) and the Enteropathogenic *E. coli* (EPEC) was the most comon group, these organisms were isolated from 33(16.5%) of the patients while *E. coli* isolates from other than EPEC were isolated from 6(3%) of the patients. The recovery rate of EPEC was 16.5% which agrees with the results of Makkia et al. (1988) and Tawfeek et al. (2002) which are 16% and 13% respectively. However, Hasony (1996) showed that enteropathogenic *E. coli* was found in 50% of examined stool specimens.

After EPEC *E. coli* isolates the second common enteropathogen found was *Salmonella* species with isolation rate of 13(6.5%). The recovery rate of *Salmonella* in our study is similar to those reported by Qadri et al. (1990) and Tawfeek et al. (2002) who mentioned that the recovery rates are 7%, 7% respectively.

In addition, *Klebsiella* species, *E. cloacae* and *M. morganii* were frequently found in this study with the isolation rates of 3.5%, 3% and 1% respectively. Among *Klebsiella*, *K. oxytoca* was the main species representing 6(85.7%) followed by *K. pneumoniae* 1(14.3%). It has also been reported that *M. morganii* and *E. cloacae* cause diarrhea in infants and children and the member of these genera have been linked to epidemics of diarrhea because some strains appear to have acquired plasmids from *E. coli* that code for the heat stable and heat labile enterotoxins (Ewing, 1986; Rasool et al., 2003).

Ability of plasmid DNA content in some isolated bacterial species to amplify their copy number:

The number of different bacterial isolates that are resistant to 15 µg/ml chloramphenicol and tetracycline are 64 and 39 respectively but not all the isolates can tolerate high concentrations of these two antibiotics. Only 20 isolates (11 *E. coli*, 3 *Klebsiella* ,3 *Salmonella*,2 *Enerobacter* and 1 *Morganella*) can tolerate 150 µg/ml of chloramphenicol and tetracycline. Then these tolerant bacterial isolates were tested for their ability to amplify their plasmid DNA content. Only two isolate of *E. coli*, two of *Klebsiella* and one isolate of *Salmonella* showed no remarkable change in their growth after addition of increased concentrations of chloramphenicol and tetracycline separately.

The results of plasmid DNA amplification in the chosen bacterial isolates are demonstrated in the following table.

Table 1 : Amplification of plasmid DNA content in chosen bacterial isolates**.**

Iso. No.	**Type of bacteria**	**Antibiotic Used**	**Growth optical density at 590 nm in presence of**		**Yield of plasmid DNA in µg/ml**		**Fold of amp.**
			15 µg/ml	**150 µg/ml**	**15 µg/ml**	**150 µg/ml**	
80	*K. oxytoca*	Cm	0.518	0.546	6.0	56.5	9.41
86	*Salmonella*	Cm	0.506	0.519	9.2	60.0	6.52
97	*E. coli*	Cm	0.586	0.600	7.0	112.0	16
31	*K. oxytoca*	Tc	0.528	0.559	4.8	33.0	6.87
90	*E. coli*	Tc	0.568	0.580	8.0	90.0	11.25

It is clear from the above table that there are remarkable increase in the values of plasmid DNA yields of the isolates numbered (80, 86 and 97) which belong to *Klebsiella oxytoca*, *Salmonella* and *E. coli* and these values of plasmid DNA yields are 56.5, 60 and 112 µg/ml respectively after growing in presence of 150 µg/ml chloramphenicol. These are notable increase of plasmid DNA yield values if they are compared with those values which obtained from the same isolates but growing in presence of 15 µg/ml chloramphenicol. Also the isolate numbered 31 (*Klebsiella* oxytoca) and 90 (*E. coli)* yield high values of plasmid DNA concentration after growing in nutrient broth containing 150 µg/ml tetracycline and they are 33 and 90 µg/ml. From the results obtained, it is clear that the chosen bacterial isolates vary in their capablities to amplify their plasmid DNA content because the folds of amplification range between 6.52-11.25.

The action of chloramphenicol or tetracycline to amplify the plasmid content in certain bacterial appear to be related to the cell cycle. Proteins which are necessary for cell division are synthesized early in the cell cycle (Kruse and Stahl, 1989). Addition of chloramphenicol or tetracycline will prevent synthesis of new proteins including host encoded DNA polymerase I (poly. I) and plasmid encoded ROP protein. Poly. I is present in excess, so even in the absence of protein synthesis there is sufficient Poly. I in the bacterial cells for many rounds of plasmid DNA replication. In contrast, Rop. protein is limiting so in the absence of new protein synthesis there is insufficient Rop protein to inhabit replication and accumulation of plasmid to copy number much greater than normally found (Datta and Hughes, 1983).

Our results are in accordance with those reported by Philippidis et al. (1991) who reported that there are overall 47 folds of amplification increase in the plasmid DNA content of *E. coli* after treatment with chloramphenicol. Also Azad et al. (1992) found that the plasmid RobI encoding beta- lactamase in *E. coli* has the ability to amplify its copy number after adding increased concentration of chloramphenicol. In addition, Ahmad and Aziz (2004) demonstrated that among *Salmonella* isolated from different clinical specimens including stool samples from patients suffering from diarrhea, three isolates showed real amplification where the concentrations of the plasmid DNA increased in a range of 3 to 13 folds after growing in nutrient broth containing 150 µg/ml chloramphenicol as they are compared with unamplified culture yields (in presence of 15 µg/ml chloramphenicol.

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