Effect of *Lactobacillus* Bacteria on the Growth of *Escherichia coli* Isolated from Infants with Amoebic Dysentery Infections

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Abstract

This study was done in Al Batoul Teaching Hospital from 25/ 9/2016 to 1/2/2017 and 200 stool samples were collected from infants suffer from amoebic dysentery diarrhea with the age less than two years. All stool samples were examined for general stool analysis, cultured on MacConkey agar, the colony characterstic were thoroughly investigated, other biochemical tests were done according to standard procedures, then diagnosed by Api20E and VITEK 2. Fifty (40%) isolates of *E. coli* were recovered. The *E. coli* isolates showed different degrees of resistance to cephalosporines so that the resistant rate to Cefixime, Cefuroxime, Ceftriaxone and Cefoxitin were 88%, 90%, 84% and 12%, respectively. The sensitive ratio of all isolates to Meropenem antibiotic was 92%. The result showed that MIC values of the Cefixime ranged between 32 and 512 µg / ml, Cefuroxime, Ceftriaxone 32 and 1024 µg / mL and Tetracyclin 16 and 512 µg / ml. The current study showed that 96% of isolates from the infant's stool have the ability to produce hemolysin, whereas 4% of the isolates produce bacteriocin with a very high significant statistical difference (P = 0.001). *Lactobacillus* bacteria were isolated from the stools of very young children and were dependent on breastfeeding by 15%, and 11.4% were obtained from the vaginal wall swabs, the colony morphology was thoroughly investigated and other biochemical tests were done for identification. One isolate was selected as probiotic to determine the inhibitory effect against *E. coli* and the diameter mean of the inhibition zone on

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50 isolates of *E. coli* was 22.82 mm. It was concluded that *Lactobacillus* bacteria are useful as probiotics for the treatment of diarrhea caused by *E. coli*.

Keywords: *E. coli*, antibiotic, diarrhea infant, *Lactobacillus.*

تأثير بكتريا العصيات اللبنية على نمو الأشريشيا القولونية المعزولة من أصابات الزحار الأميبي في الأطفال الرضع

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الخلاصة

أُجريت هذه الدراسة في مستشفى البتول التعليمي للفترة 25 /9 /2016 ولغاية 1 /2 /2017، تضمنت الدراسة جمع 200 عينة براز من أطفال بعمر سنتين فما دون مصابين بأسهال الزحار الأميبي، وزرعت النماذج المرضية على وسط الماكونكي الصلب، تم عزل وتشخيص 50 عزلة تنتمي لبكتريا الأشريشيا القولونية بالأعتماد على الصفات المظهرية والفحوصات الكيموحيوية وبأستخدام نظامي التشخيص Api20E و VITEK 2. أُجري إختبار حساسية العزلات لخمسة عشرة مضاداً حيوياً وأظهرت العزلات مقاومة عالية لمضادات مجموعة السيفالوسبورينات حيث بلغت نسبة المقاومة للمضادات Cefixime، Cefuroxime، Ceftriaxone و Cefoxitin 88%، 90%، 84% و12% على التوالي بينما كانت حساسية العزلات عالية لمضاد Meropenem (92%). تراوحت قيم التركيز المثبط الادنى (MIC) لمضاد Cefixime تراوحت بين 32 _512 مايكروغرام / مل، Cefuroxime وCeftriaxone 32_1024 مايكروغرام / مل، أما مضاد Tetracyclin فقد تراوحت قيم MIC له من 16_512 مايكروغرام / مل. كما أُجري الكشف عن إنتاج العزلات الجرثومية لعوامل الضراوة التي شَمِلت أنتاج الهيمولايسين والبكتريوسين حيث أنتجت البكتريا المعزولة من براز الأطفال الرضع الهيمولايسين بنسبة 96% والبكتريوسين بنسبة 4% وبفارق أحصائي معنوي عالي جداً (P =0.0001). تمَّ عزل بكتريا *Lactobacillus* من براز الأطفال الأصحاء بعمر عدة أيام والمعتمدين على الرضاعة الطبيعية بنسبة 15% و من جدار المهبل بنسبة 11.4% وشخصت بالاعتماد على الصفات المظهرية والأختبارات الكيموحيوية، أستخدمت أحدى العزلات كمعزز حيوي لمعرفة تأثيرها التثبيطي على بكتريا أشريشيا القولون حيث بلغ معدل قطر منطقة التثبيط لبكتريا *Lactobacillus* على (50) عزلة من بكتريا أشريشيا القولون بلغ 22.82 ملم، مما يستنتج امكانية الأستفادة من العصيات اللبنية كمعزز حيوي لعلاج حالات الأسهال لدى الرضع

الكلمات المفتاحية: اشريشيا القولون، أسهال الاطفال الرضع، المضادات الحيوية، العصيات اللبنية.

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Introduction

Diarrhea is a symptom caused by gastrointestinal dysfunction or a change in the normal nature of stool in terms of the number of times of defecation (more than five times within 24 hours) with liquid or semi-liquid strength, resulting in loss of fluid [1]. Diarrhea occurs due to the exposure of human intestines to several types of pathogenic microorganisms such as *Rotaviruse, Entamoeba hystrolytica, Candia albican* as well as bacteria such as *Escherichia coli, Salmonella and Shigella* etc. Each of these microbial pathogen causes a disease in a different mechanism [2]. *Escherichia coli* is a normal flora where it is present in the human intestine and mammals. However, some strains have the characteristics of virulence, causing many diseases, which have been described as a cause of diarrhea in children.

The enteropathogenic *E. coli* (EPEC) is a pathogen causing diarrhea, especially in children aged less than 5 years in developing countries and the infection is associated with one or more of the parasitic and viral pathogens of gastroenteritis such as

E. histolytica and *Balantidium coli* [3]. Gastrointestinal bacteria are formed naturally in the gut after several days of birth. *Escherichia coli* is the main part of the gut and when these bacteria leave the original location (bowel) and move to other organs such as the urinary canal and the bile duct become opportunistic, especially when appropriate conditions such as immune impairment are available [4].

The emergence of resistant strains to antibiotics led to fears that drug factories could not produce effective drugs to counteract the emergence of these strains therefore, there is a need to consider using other methods of treatment, especially probiotic, which promote the colonization of normal flora in the intestines and thus, protect against diarrhea and other diseases [5]. Studies have indicated the use

of *Lactobacillus* bacteria in the treatment of cases of diarrhea caused by various intestinal infections, because this type of bacteria have protective effects against intestinal infections [6].

The current study aims to conduct general stool analysis for infants infected with diarrhea associated with amoebic dysentery, isolation of *E. coli* from infants aged two year old or less,

isolation and identification of *Lactobacillus* and comparing their inhibitory effect with selected antibiotics used in the study.

Materials and Methods

Specimens Collection

In this study, 200 stool specimens were collected from infant suffering from amoebic dysentery diarrhea (aged less than two years) from Al Batoul Teaching Hospital during the period 25/ 9/2016 to 1/2/2017.

Isolation and Identification of *E.coli*

Stool samples from infants infected with diarrhea were examined for general stool analysis, cultured on MacConkey agar and Eosin Methylene Blue (EMB), All isolated were identified on the basis of colony characteristics, Gram stain, biochemical tests, ApiE20 and confirmation of identification were achieved by using the VITEK 2 (Biomeriex- France) [7]. Hemolysin and bactericin production were also studied according to [8] [9].

Antibiotic Sensitivity Test

Antibiotic susceptibility test of *E. coli* isolates was determined by the standard Kirby-Bauer disk diffusion method [10], fifteen antibiotics were used included Chloramphenicol (30 µg), Amikacin (30 µg), Cefixime (5 µg), Cefuroxime (30 µg), Ceftriaxone (30 µg), Cefoxitin (30 µg), Ciprofloxacin (5 µg), Gentamycin (10 µg), Augmentin (20_10 µg), Piperacillin (100 µg), Ticarcillin (75 µg), Meropenem (10 µg), Ampicillin_ Sulbactam (10_10 µg), Doxycycline (30 µg), Tetracycline (30 µg). Results were expressed susceptible or resistant according to the criteria recommended by the CLSI [11].

MIC test: The minimum inhibitory concentrations (MIC) of the Cefixime, Cefuroxime, Ceftriaxon and Tetracyclin were determined by serial double dilutions technique [12].

***Lactobacillus* bacteria**

Collection of sample:

20 stool sample were collected from breast-fed infants**,** 35 vaginal swabs were collected from women of different ages. The samples were collected from Al Batoul Teaching Hospital, Baquba, Diyala provice, Iraq.

Isolation and identification of *Lactobacillus*

About one gram of each stool sample was cultured in DeMan, Regosa, Sharp broth and incubated under anaerobic condition at 37˚C for 48h, then subcultured on MRS agar and incubated under anaerobic condition at 37˚C for 48h. Growth was streaked on MRS agar plates several times [13], vaginal samples were collected from vaginal wall were inoculated on MRS agar and inocubated overnight anaerobically at 37 ºC for 48h, growth was streaked on MRS agar plates several time [14].Identification of isolated bacteria as *Lactobacillus* was performed according to their morphological, cultural and biochemical characteristics according to procedures described by [15]. Study of the effect of some factors on the growth of *Lactobacillus* include:

A-Assay for NaCL tolerance:

NaCL tolerance of isolated *Lactobacillus* were determined by procedures as described in [16].

B-Assay for phenol tolerance:

Phenol tolerance of isolated *Lactobacillus* were determined by procedures as described in [16].

C-Assay for bile salts Tolerance:

Bile salts tolerance of isolated *Lactobacillus* were determined by procedures as described in [17].

D-Determination of optimum pH for growth: The ability of *lactobacillus* bacteria to grow at different levels of pH and determination of optimum pH for growth by procedures as described in [18].

Detection of antimicrobial activity:

well diffusion assay method was used for the detection of antimicrobial activity of *lactobacillus* on the growth of *E.coli* as described in [19].

Statistical analysis

Data were analyzed by using Chi-square to compare between percentages and T test to compare between numeric data (means ±SD). Duncan test also has been used to compare between means of numeric data at level of significance of P≤ =0.05 was applied to test. SPSS (v. 21) and Excel 2013 programs were used to analyze current data [20].

Results and Discussion

In this study, *E. coli* was isolated from 200 stool sample from infants suffering from diarrhea associated with amoebic dysentery. A general stool test was performed and then culture on MacConky agar and 50 (41%) isolates of *E. coli* were obtained. The results of the biochemical tests of *E. coli* showed that all these isolates yielded positive results for Catalase, Methyl red and Indol, while negative results with Voges-Proskauer (VP), Citrate Utilization and Oxidase. The API_20E and VITEK 2 were then used. The results were compatible with the results of the biochemical tests, all isolates were confirmed to be *E. coli* bacteria.

Table 1: Distribution of *E. coli* isolates on the base of age and sex from infants suffering from diarrhea associated with amoebic dysentery.

Age group	NO.	%	Sex	NO.	%
(1-6) months	20	40	Males	30	60
(12-7) months	18	36	Females	20	40
(13-24) months	12	24	Total	50	100
Total	50	100	X^2	2.13	
X^2 P	9.72 0.007 [NS]		P	0.15 [NS]	

The highest rate of *E. coli* isolation (40%) was found in the age group (1-6 months) while the lowest rate (24%) was found in the age group (13-24 months), and the result did not show any significant differences which agreed with the result of Gomes et al [21]. This study showed that

there were no significant differences between male and female infants infected with *E. coli*, so that the percentages of *E. coli* in males and females were 60% and 40%, respectively. This result was similar to the result of Nweze [22] (Table 1).

Table 2: Distribution of *E. coli* isolates on the base of housing and type of feeding.

Feeding type	NO.	%	Housing area	NO.	%
Breast feeding	6	12	Rural	27	54
Bottle feeding	38	76	Urban	23	46
Mixed feeding	6	12	Total	50	100
Total	50	100	X^2	0.32	
X^2 P	45.47 0.001 [S]		P	0.57[NS]	

These bacteria were found in a larger proportion of children and were dependent on bottle feeding with a prevalence rate of 76% and with a very high significant difference (P = 0.001) and this finding agreed with the finding of Ali et al. [23] where they found that the proportion of *E. coli* infected infants depended on breast feeding was lower than bottle and mixed feeding. There were no significant differences in infection of *E. coli* associated with housing (Table 2). Bactericin has a great medical importance as it is produced by non-pathogenic bacteria that normally found in the human body [24]. This study revealed that low number of *E. coli* isolates (4%) produced Bactericin. This percentage is very low and this may be because the isolated bacteria are pathogenic and Bactericin is produced by non-pathogenic bacteria, whereas 96% of isolates were appeared to have the ability to produce hemolysin with a very high significant difference (P = 0.001) between the isolates. Resistance rate of *E. coli* isolates to Cefixime, Cefuroxime and Ceftriaxone so that the resistant rate were 88%, 90% and 84%, respectively. However, 98% of isolates were resistant to Ticarcillin, 56% resistant to Tetracycline, whereas the sensitivity ratio of isolates to Chloramphenicol, Amikacin, Cefoxitin, Ciprofloxacin, Gentamycin, Augmentin, Piperacillin, Meropenem, Ampicillin, Sulbactam and Doxycycline were 62%, 70%, 78%, 56%, 64%, 54%, 70%, 92%, 56%, 58%, respectively (Table 3).

Table 3: Sensitivity of *E. coli* to (15) antibiotics

Antibiotics	Resistance (number and %)	Sensitivity (number and %)
Chloramphenicol	9 (18)	31 (62)
Amikacin	6 (12)	35 (70)
Cefixime	44 (88)	3 (6)
Cefuroxime	45 (90)	3 (6)
Ceftriaxone	42(84)	3 (6)
Cefoxitin	6 (12)	39 (78)
Ciprofloxacin	10 (20)	28 (56)
Gentamycin	13 (26)	32 (64)
Augmentin	10 (20)	27 (54)
Piperacillin	8 (16)	35 (70)
Ticarcillin	49 (98)	1 (2)
Meropenem	0 (0)	46 (92)
Ampicillin_ Sulbactam	12 (24)	28 (56)
Doxycycline	15 (30)	29 (58)
Tetracycline	28 (56)	22 (44)
X^2	**402.46**	**247.47**
P	**0.001[S]**	**0.001[S]**

The results of the minimum inhibitory concentrations (MIC) of the Cefixime, Cefuroxime, Ceftriaxone and Tetracyclin showed that values of MIC were ranging between 16 and 1024 ug/ml **(**Table 4).

Table 4: The minimum inhibitory concentration values of Cefixime, Cefuroxime, Ceftriaxone and Tetracycline antibiotics.

Isolate no.	CFM	CXM	CRO	T
	≥ 4	≥ 32	≥ 4	≥ 16
1	512	1024	256	64
2	256	32	512	64
3	512	256	64	32
4	512	32	256	32
5	512	32	256	64
6	256	32	256	64
7	64	128	256	64
8	64	128	1024	512
9	128	512	32	512
10	32	512	512	32
11	256	512	512	32
12	512	256	512	16
13	128	128	512	512
14	128	512	512	64
15	512	1024	64	128
mean ± SD	197.58 ± 292.26	336.19± 341.33	336.19 ± 369.06	191.13 ±146.13
T test	5.33	3.77	5.62	2.99
P	0.001[S]	0.002[NS]	0.001[S]	0.01[S]

Cefixime (CFM), Cefuroxime (CXM), Ceftriaxone (CRO), Tetracycline (T)

This study showed that the proportion of *Lactobacillus* bacteria (LA) isolated from the stools of the infants was 15% while *Lactobacillus* isolates from vaginal swabs (LB) was 11.4%. They were identified as *Lactobacillus* depending on the morphology and biochemical characterization and the results of the biochemical tests of *Lactobacillus* showed that all isolates (7 isolates) yielded negative results for Catalase, Oxidaes, Indole, Citrate utilization, non-motile and non Gelatine hydrolysis. Sugar fermentation patterns of the isolates indicated that Maltose, Lactose, Sucrose and Glucose fermented by isolated *Lactobacillus*, Arabinoseand Sorbitole were non-fermented. The effect of several factors on their growth was studied such as pH, NaCL, bile salt and 0.4% phenol. pH is an important and influential factor in the growth of *Lactobacillus*, the study showed that growth of isolated bacteria occurred within the pH range from 4-7, but the optimum pH value for growth was at pH 6.5 and these bacteria were not able to grow when the pH increased to 8 and similar results were observed by Pyar and Peh [15].

In the present study, it was observed that *Lactobacillus* isolates were able to grow in different concentrations of sodium chloride solution, but when the concentration increased, a significant reduction was observed in their growth as they did not grow at concentrations of 8% and 9%. This finding was agreed with Elizete and Carlos [25] where they found that they had the potential to grow at concentrations ranged between 2 and 7% (Table 5).

Table 5: Tolerance of *Lactobacillus* isolates for NaCL and different levels of pH.

Parameter1	pH of medium								
	4		5	6		6.5	7	8	
Growth	+		+	+		+	+	-	
Parameter2	NaCL concenteration (%)								
	1%	2%	3%	4%	5%	6%	7%	8%	9%
Growth	++	++	+	+	+	+	+	-	-

No Growth (-), Growth (+), Good growth (++)

The result revealed that *Lactobacillus* bacteria were able to grow at a concentration of 0.4% phenol. Five out of seven isolates showed growth potential in the of 0.4% phenol, the results are in agreement with finding of Elizete and Carlos [25]. The resistance to bile salts is an important criterion in the selection of microorganisms used as probiotics [26]. This finding revealed that isolated *Lactobacillus* were able to maintain their growth well at concentrations (0.2%, 0.4%) of bile salts, especially LB1, LB2, and LA7 isolates whereas other isolates showed a significant decrease in growth when the concentration increased to 0.4 %. Similar results were observed by Sahadeva et al [27] (Table 6).

Table 6: Tolerance of *Lactobacillus* isolates to bile salts and phenol.

Parameter3	Bile salt		Parameter 4	Phenol
Isolate no.	0.2%	0.4%	Isolate no.	0.4%
LB1	+++	+	LB1	+
LB2	++	+	LB2	+
LB3	+	-	LB3	-
LB4	+	-	LB4	-
LA5	+	+	LA5	+
LA6	+	+	LA6	+
LA7	+++	++	LA7	+

No Growth (-), Growth (+), Good growth (++), Very good growth (+++)

In this study, the tolerance of *Lactobacillus* for several factors was tested, LA7 isolate from the infant stools was able to tolerate a wide range of pH and different concentrations of bile salts and therefore, it was used as a probiotic to determine its inhibitory effect on *E. coli* bacteria. The results of antagonistic effects revealed that *Lactobacillus* isolated from infant 's stool was effective against pathogenic isolates of *E. coli* and had the best effect as evidenced by the diameter of the inhibition zone (26 mm), while the mean diameter of the *Lactobacillus* inhibition zone on 50 isolates of *E. coli* was 22.82 mm. These results are in agreement with those reported by Nasif et al. [28] and it was reported that the inhibitory activity of *Lactobacillus* was due to its to produce of bactericin, which acts as an antibiotic [29].

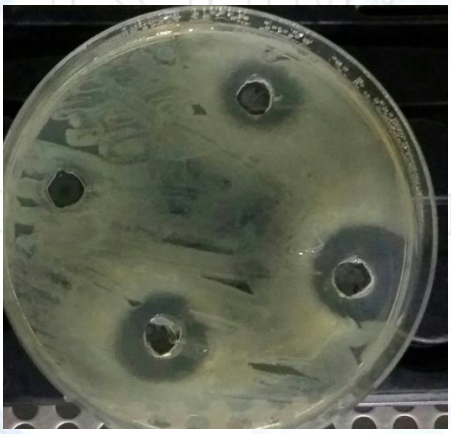

Figure 1: The inhibitory effect of *Lactobacillus* bacteria against *E. coli*.

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