University of Thi-Qar Journal of Science (UTJsci)
E-ISSN:2709-0256, ISSN Print: 1991-8690 Volume (10), No.1, June 2023

Isolation and identification of Gram negative bacteria that cause diarrhea

Riyam Al-Hilali
Department of Biology / College of Science/ University of Thi-Qar
Iraq
riyamm@sci.utq.edu.iq

Hanaa Al-Mozan
Department of Biology / College of Science/ University of Thi-Qar
Iraq
hanaa.d@sci.utq.edu.iq

***Abstract*—: Diarrhea is an essential contributor to morbidity and mortality in all parts of the world and among people of all ages. Fecal-oral transmission and consumption of food and water tainted with pathogenic organisms are the leading causes of acute infectious diarrhea, according to studies. this study was conducted to isolate some types of bacteria that cause diarrhea in humans, and 350 stool samples were collected from all ages of both sexes who suffer from diarrhea in Al-Shatrah General Hospital, Bint Al-Huda Hospital, and the Public Health Laboratory in Thi-Qar province during the period from October 2022 to January 2023. Morphological traits routine and advanced biochemical tests were adopted in this study, The results showed that the percentage of bacteria isolates that cause diarrhea was (5.71%), *Salmonella spp* (4) (20%), *Aeromonas* spp (2) (10%), *Enterobacter* spp(14) (70%). *Enterobacter* spp had the highest rates of resistance to Cefotaxime/clavulanic acid (28.571%), while *Aeromonas* spp had the highest rates of sensitivity to the same antibiotic (50%). Enterobacter bacteria were the most infected in cases of diarrhea; this work showed that the anti-amikacin is the best in treating cases of diarrhea, and the anti-amoxicillin-clavulanic acid is the antibiotic that is characterized by high resistance to bacteria.**

***Keywords*— Diarrhea, API 20 E, Antibiotics resistance**

I. INTRODUCTION

Diarrhea is a major cause of morbidity and mortality in all regions of the world at among all ages. More than 2 million people died yearly, especially infants under five years old, because of diarrhea[1]. The main causes of diarrhea are wide range of viral bacteria and parasitic pathogen, and that varies depending on differentof factors such as geographic and climate conditions, host factors, and socioeconomic situations [2].

There are various methods for isolating and identifying bacteria, One of these method is Gram stain that has been used to differentiate between Gram-negative and Gram-positive bacteria. Christian Gram proposed this technique to discriminate between two species of bacteria based on differences in their cell wall architecture. Gram-positive bacteria retain the crystal violet dye due to a thick layer of peptidoglycan in their cell wall. This method differentiates bacteria by recognizing peptidoglycan in the cell walls of gram-positive bacteria. When gram-negative bacteria are exposed to alcohol, a very thin layer of peptidoglycan dissolves[3-4].

The using of some methods to diagnose in bacterial diarrhea is very important to decrease any more severe conditions and having sever symptoms. It is critical to understand this pathogen and distinguish it from other, The majority of bacterial diarrhea cases occurred due to foodborne. In situations of bacterial diarrhea, clinical decision-making includes deciding when to do diagnostic stool testing and when to treat with antibiotics [5].

The majority of infectious diseases are caused by bacteria. The discovery of laboratory methods to grow these microorganisms using an appropriate growth medium known as culture is essential for healthcare providers to determine immediately an appropriate treatment for their patients [6]. With increasing reports of increased antibiotic resistance among gut bacteria, treating bacterial diarrhea will be difficult. Reports of more than 40% of non-bacterial diarrhea cases in children are being treated with antibiotics. There are some studies showed that the role of bacteria in diarrhea cases with a prevalence rate of 3% and high resistance to commonly used drugs [2]. In addition, If it is a bacteria type that does not have uniform sensitivity to antibiotics, the antibiogram should also be reported, since the significant increase in antimicrobial resistance represents an obstacle to empirical treatment in some cases.

II. MATERIALS AND METHODS

A. *Collection of samples*

Three hundred fifty of stool samples from patients, male and female,with diarrhea were collected from Shatrah General Hospital, Bint Al Huda Hospital, and Public Health Laboratory in Thi-Qar province during the period from October 2022 to January 2023. Stool samples were collected directly into a sterile tube containing peptone water, and immediately transfer it to the bacteriology laboratory of Shatrah General Hospital with a cool box [7].

B. *Culturing of specimens*

Fecal samples were incubated on peptone water for 24 hr at 37 °C, then cultured directly on MacConkey agar and

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https://doi.org/10.32792/utq/utjsci/v10i1.1059

subcultured on Tetrathionate broth with iodine solution(selective for *salmonella*) for 24 hr at 37 °C. After that,the samples were transferred from Tetrathionate broth into Xylose Lysine Deoxycholate XLD medium and incubated at 37 °C overnight (18–24 hr). Following that, MacConkey and XLD agar plates were examined. The diagnosis was then made by tracing bacterial morphology and using biochemical confirmation panels.

A gram stain was made for the colonies growing on the ingredient MacConkey agar, , and then biochemical tests were done to look for bacteria other than *Salmonella* [7].

C. Biochemical tests

The important biochemical tests were conducted according to [8]. Tests Kligler iron (KI), Oxidase test, Lactose fermentation, Urease test, Indole test, Citrate utilization test.

D. Analytical profile index for Enterobacteriaceae test(API 20 E) for Isolate Identification

According to (BioMerieux, France), this test is used clinically for the rapid identification of Enterobacteriaceae. This test consists of a strip that contains 20 small tubes with an upper and lower orifice (cupule and tube) containing dried material and representing a biochemical test. Color changes occur in the tubes during incubation or after the addition of the reagents.

E. Antibiotic Susceptibility Test

The antibiotic susceptibility test was carried out using the disc diffusion technique in accordance with the clinical laboratory standards institute (CLSI) criteria. Antibiotic susceptibility to diarrhea-causing bacteria isolates has been detected using a variety of antibiotic disks. The antibiotic disks used in this study are shown in Table 1.

TABLE 1. the antibiotic disks are used in the current study

No.	Antibiotic	Symbol	Concentration µg.
1	Amoxyclav(Amoxicillin-clavulanic acid)	AMC	30
2	Cefotaxime/clavulanic acid	CEC	30/10
3	Tetracycline	TE	30
4	Amikacin	AK	30
5	Piperacilline	PI	100

III. RESULTS AND DISCUSSION

1) Isolation and Identification of Bacteria

A total of 350 samples, only 20 samples (5.71%) were isolated Fig.1. , the percentage of bacterial diarrhea in this study corresponds to this percentage [9]. He was able to isolate six out of 153 stool samples (4%).

The samples included 4(1.14%) *Salmonella.* The percentage was low, which agrees with [10], who isolated 297 (5.7%) Salmonella out of 5239 patients. These rates of decline may be due to the extent to which antibiotics affect bacteria.

The incidence of *Aeromonas* was low at 2/350 (0.57%), which agrees with [11], who isolated 17 (2%) of the 1,033 samples. While disagrees with [12], among the 216 stool samples tested, 21 (9.7%) were positive for *Aeromonas.* This difference is due to the presence of the egg masses of chironomids, non-biting midges (Diptera: Chironomidae), and that acts as a natural reservoir for the pathogenic species of *Aeromonas* [11]. The use of alkaloid media may have resulted in the availability of a suitable laboratory environment for bacteria.

The results of bacterial cultures obtained in this study showed that the total range of *Enterobacter* that were isolated from stool were14/350 (4%).These findings agrees with [13], who scored (6%), and disagrees with [14] who reported higher percentage (30.76%), The differences in the prevalence of Enterobacter isolates with the previous study could be attributed to multiple factors, such as geographic and seasonal variation, sample procedure management practices, and sanitary conditions or due to differences in the sensitivity and specificity of the different isolation methods used.

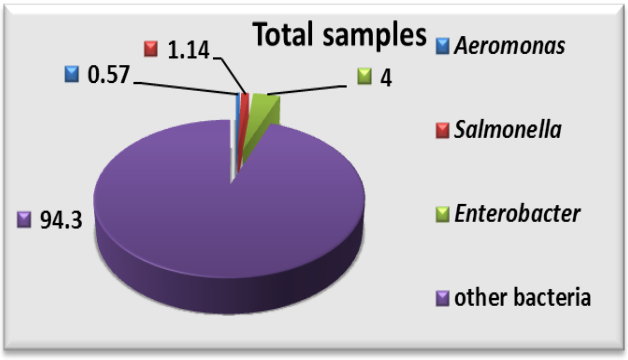


Fig.1. bacteria are isolated from patients

A. Morphological properties

The results showed different morphological characteristics of the bacterial genera in this study where it appeared Salmonella on Xylose-Lysine Deoxycholate agar (small, smooth, rounded, red in color with a black center). Fig.2. *Aeromonas* on MacConkey agar as a non-lactose fermenter Fig.3. Enterobacter on MacConkey agar as a lactose fermenter with pink-mucoid colonies on Fig.4.

Fig.2. *Salmonella* spp. at 37 °C for 24 hrs. Growth on Xylose Lysine Deoxycholate (XLD) Agar

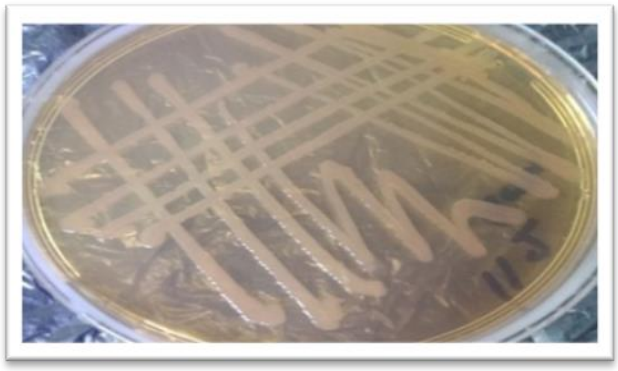


Fig.3. *Aeromonas* spp. at 37 ℃ for 24 hrs. Growth on MacConkey agar

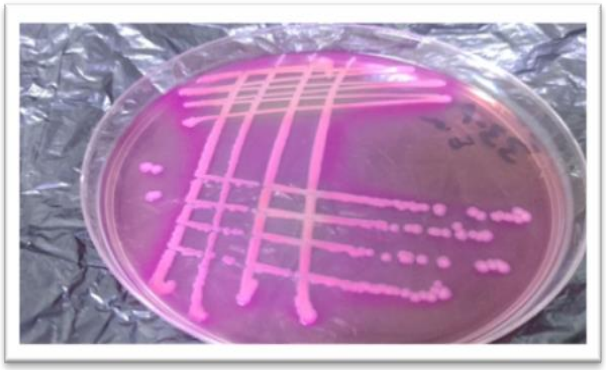


Fig.4. *Enterobacter* spp. at 37 ℃ for 24 hrs Growth on MacConkey Agar

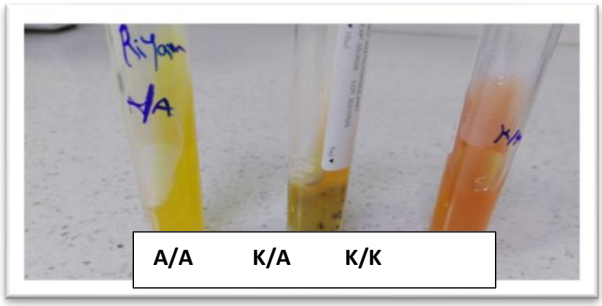
B. Biochemical tests

The results of the biochemical tests showed that these isolates gave negative results for the oxidase and indole tests, except for, *Aeromonas* which was positive, and urease was also negative, while it gave positive results for the citrate test, except for *Aeromonas*, which was positive, as shown in Table 2 and Fig. 5.

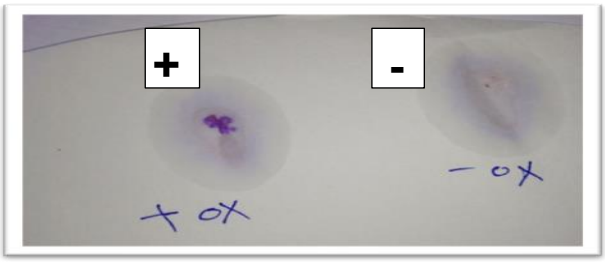
TABLE 2. The results of some biochemical tests of Gram negative bacteria

Biochemical test	**Result**		
	Salmonella spp	*Aeromonas spp*	*Enterobacter spp*
Kligler iron (KI)	Red/Yellow (K/A)with H2S production With or without gas production	Red/Yellow(K/A) with gas*v	Yellow /Yellow (A/A)
Oxidase test	-	+	-
Lactose fermentation	Non-lactose fermenter	Non-lactose fermenter	lactose fermenter
Urease test	-	-	-
Indole	-	+	***v**
citrate utilization test	(+) for Salmonella (majority) and (-)for Salmonella Typhi	-	+

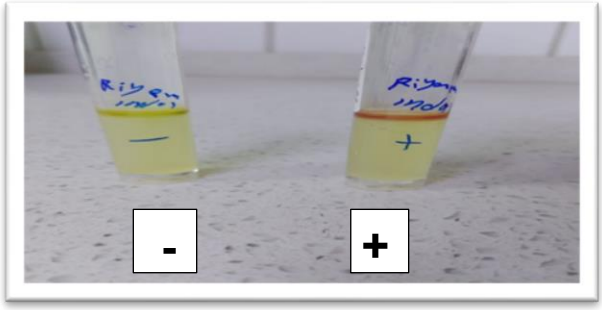
***v:** *variable(**– and +)**



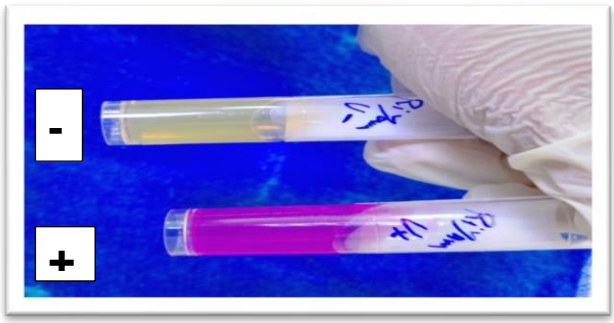
(A) Kligler iron



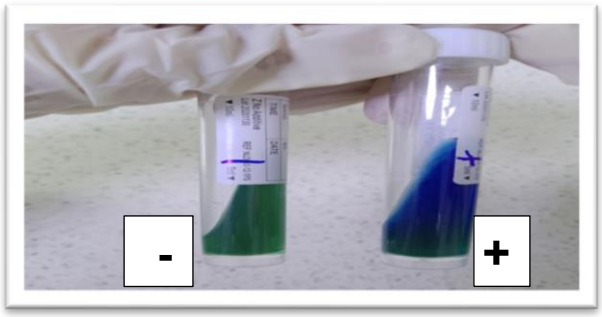
(B) Oxidase test



(C)Indol



(D) Urease test

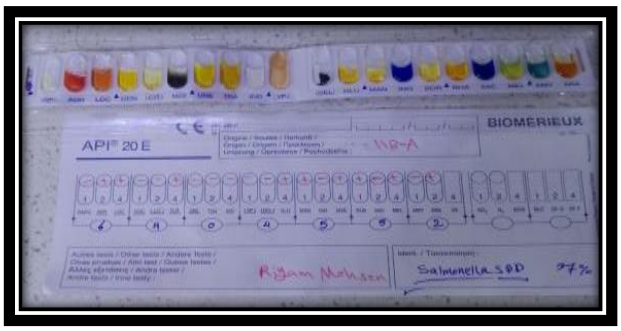


(E) citrate utilization test

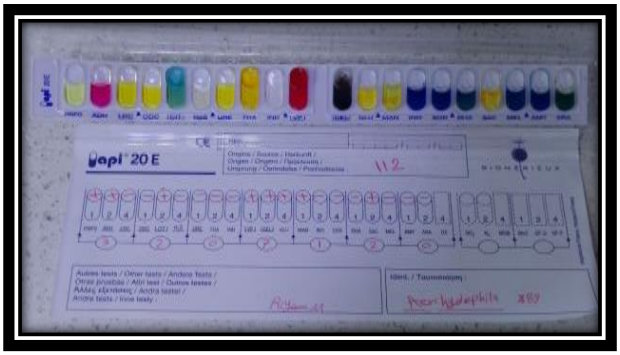
Fig.5. Biochemical tests: (A) Kligler iron, (B) Oxidase test, (C)Indol, (D)Urease, , (E) Citrate utilization.

C. Confirmation of bacteria by API 20E system

Analytical profile index for Enterobacteriaceae test was used to confirm the identification of all the isolates. Fig.6. shows the results from 20 isolates of *Salmonella* , *Aeromonas* , and *Enterobacter.*



A: *Salmonella spp*



B: *Aeromonas spp*

C: *Enterobacter* spp

Fig.6. Results of Examination by API 20E System where: (A) *Salmonella*, (B)*Aeromonas*, and (C) *Enterobacter*.

2) Multi-drug Resistance Pattern of Bacteria that are isolated from Stool Samples

This study shows that All *Salmonella* were Amikacin sensitive, which agrees with[15]. The reason may be due to its great effectiveness against Gram-negative bacteria and its ability to treat a wide spectrum of bacterial diseases. A high frequency of resistance was found to piperacillin (100%) and Amoxicillin-clavulanic acid (100%), This agrees with[16], [10], and[17] where amoxicillin-clavulanic acid (96%), piperacillin (64.5%), and amoxicillin (82.89%), The cause is due to the overuse of antibiotics It is the main accelerator of the emergence of resistance [18]. The percentage of *Salmonella* isolates resistant to Tetracycline (15%) differs from the percentage of findings (63.5%) reported by [10]. *Salmonella* resistance to cefotaxime is 25%, which disagrees with the results of [16]that found resistance of cefotaxime to be 89%, Perhaps the explanation for the resistance is that antibiotics were used indiscriminately and untargetedly throughout the Covid-19 period, leading to a surge in antibiotic resistance. Table 3. Fig.7and 8.

TABLE 3. Percentage of *Salmonella* resistant to some antibiotics

Antibiotic	**Resistance**		**Intermediate**		**Sensitive**	
	No	%	No	%	No	%
Amoxyclav(Amoxicillin-clavulanic acid)	4	100 %	0	0.0	0	0.0
Cefotaxime/clavulanic acid	1	25%	3	75%	0	0.0
Tetracycline	1	25%	1	25%	2	50%
Amikacin	0	0.0	0	0.0	4	100 %
Piperacilline	4	100 %	0	0.0	0	0.0

Aeromonas species are known to be intrinsically susceptible to all antibiotics active against non-fastidious Gram-negative bacilli, except for many beta–lactams, due to the production of multiple inducible, chromosomally encoded β–lactamases[11], All *Aeromonas* isolates are Amikacin sensitive, which agrees with [11], [19] . *Aeromonas* were100%resistant to Amoxicillin-clavulanic acid and piperacillin. This disagree with [11], who reported (46%) for Amoxicillin-clavulanic acid. The percentage of Tetracycline resistance is 50%, which agrees with[12] and [19], who recorded the percentage of resistance (71.4%) and (33%) for Tetracycline ,*Aeromonas* were 50% resistant to cefotaxime, which disagree [12]and [19] He found that there is no resistance to this antibiotic. Bacterial development and genetic mutations might causes them to be more resistant to antibotics. Table 4 and Fig.7and 8.

TABLE 4. Percentage of *Aeromonas* resistant to some antibiotics

Antibiotic	**Resistance**		**Intermediate**		**Sensitive**	
	No	%	No	%	No	%
Amoxyclav(Amoxicillin-clavulanic acid)	2	100%	0	0.0	0	0.0
Cefotaxime/clavulanic acid	0	0.0	1	50 %	1	50%
Tetracycline	0	0.0	1	50 %	1	50%
Amikacin	0	0.0	0	0.0	2	100 %
Piperacilline	2	100%	0	0.0	0	0.0

The present study of *Enterobacter* isolates has shown that all of the isolates (100%) were sensitive to Amikacin. The result of this study agrees with [20].Our results also showed that all isolates (100%) were resistant to Amoxicillin-clavulanic acid, and this percentage agrees with [14] who got (100%), and also agrees with [21] who reported (93.3%) for Amoxicillin- clavulanic acid. Piperacillin resistance was (92.9%) , and that agrees with [22] who found the resistance of the bacteria to piperacillin was 42%. Resistance to cefotaxime clavulanic acid in the current study was (28.6%).This percentage agrees with [20] and [21] who reported 26%and (33.3%) for cefotaxime clavulanic acid respectively . As for the rate of tetracycline resistance (14.3%), and this agrees with[21], as it had a resistance rate (40.0%). The results of this study might be tothe fact that in less

developing countries, people use self-medication and unprescribed drugs Lead to increasing in bacterial resistance to different antibotics. Table 5. and Fig.7and 8.

TABLE 5. Percentage of *Enterobacter* resistant to some antibiotics

Antibiotic	**Resistance**		**Intermediate**		**Sensitive**	
	No	%	No	%	No	%
Amoxyclav(Amoxicillin-clavulanic acid)	14	100%	0	0.0	0	0.0
Cefotaxime/clavulanic acid	4	28.571 %	7	50%	3	21.428%
Tetracycline	2	14.285 %	6	42.857%	6	42.857%
Amikacin	0	0.0	0	0.0	14	100%
Piperacilline	13	92.857 %	1	7.142 %	0	0.0

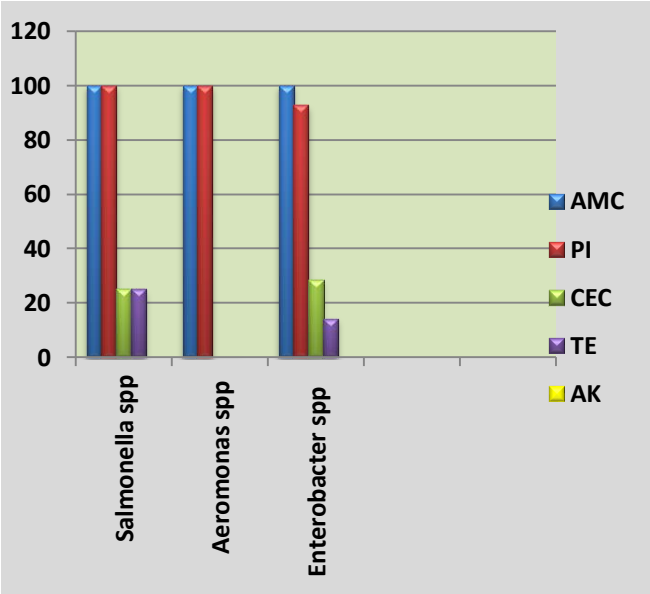


Figure.7. Resistant rate in *Salmonella , Aeromonas , and Enterobacter* isolates

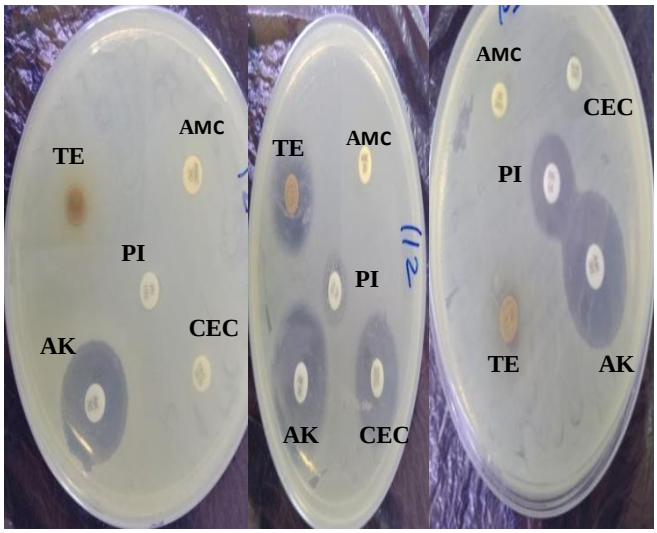


Fig.8. Susceptibility test of bacteria to some antibiotics

IV. CONCLUSION

Enterobacter bacteria were the most infected in cases of diarrhea; this work showed that the anti-amikacin is the best in treating cases of diarrhea, and the anti-amoxicillin-clavulanic acid is the antibiotic that is characterized by high resistance to bacteria.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

ACKNOWLEDGMENT

Thanks to everybody who supported us and made it possible for us to gather data for our study.

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