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Isolation and molecular characterization of *Vibrio fluvialis* from diarrheal children in Nasiriyha City

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Abstract:

The objective of this study was to investigate the incidence, molecular features, as well as the antimicrobial susceptibilities of *Vibrio.fluvialis* isolated from diarrheal (children) in Nasiriya city from (July 2016 to February2017) . A total of (20) samples of *Vibrio. fluvialis* were collected and analyzed by bacteriological, molecular and antimicrobial methods. Green and yellow bacterial colonies were recovered on thiosulfate citrate bile salt sucrose agar plates. The phenotypic characteristics of the isolates, including morphological, physiological and biochemical traits were determined and confirmed using API 20E system. Following isolation, the 16S rRNA gene specific for the genus *Vibrio.fluvailis* was investigated by using PCR. Antimicrobial susceptibility to 10 antimicrobial agents was determined by the disc agar-diffusion method .In this study the isolates were more resistance to polymaxin B (70%) & streptomycin (65%) and tetracycline (60%),while doxycycline was (60%) ,ampicillin and chloramphenicol (55%) ,while trimethoprim was resistance (50%).while was more sensitative to Nalidixic acid &Azithromycin (35%)and ciprofloxacin (45%).

The study showed an increase presences of *Vibrio.fluvialis* in diarrheal children &ahight resistance of many antimicrobial agent that importance to find suitable methods to control the infection transmission.

Key words: *Vibrio.fluvialis* **,** diarreahal, Molecular characterization.

1.Introduction:

The vibrionaceae (vibrios) are a group of straines with the following characteristics : they are Gram- negative rods with a polar flagellum enclosed in a sheath, facultative anaerobic metabolisms, are capable of fermenting D-glucose and grow at 20 C(Sawabe, *et al* ,.2013).

Addidation , most vibrio spp. ferment a variety of carbohydrates without gas production and grow on thiosulfate citrate bile salt sucrose (TCBS) agar medium (Thomposon *et al* ,. 2006).

Currently, *V.fluvialis* has an infectious importance because its clinical symptoms of gastroenteritis are very similar to that of *V. cholerae*., the emerging etiologic agent *V. fluvialis* has caused sporadic cases and outbreaks of diarrhea in several countries (Hug *et al*,. 1980& Kobayashi *et al* ,. 1989)**.** *V. fluvialis* infections are common in areas that have high levels of fecal contaminated water, food supplies and consumption of raw seafood or contaminated seafood products *V. fluvialis* infections are common in areas that have high levels of fecal contaminated water, food supplies and consumption of raw seafood or contaminated seafood products (WHO.,2009). Human-induced climates changes creating favourable conditions such as water, temperature, nutrient concentration and plankton production, for the growth and reproduction of the bacterium (Lee, *et al*,. 2001).

The conventional standard microbiological method is based on phenotypic identification, which requires several days to carry out the enrichment step, cultivation and biochemical tests(Binsztein *et al* ,. 2004). Some *Vibrio* spp. can cause problems owing to variability in biochemical characteristics within species(Lipp, A. Huq and. Colwell, 2002) A molecular biological method, such as polymerase chain reaction (PCR), is more rapid, sensitive and specific than standard culturing methods for detection of low microbial concentrations and detection of VBNC pathogens(Kaysner,. and Angelo DePaola, 2004).

An increase in the emergence of multi-antibiotics resistant bacteria in recent years is

worrisome and the presence of antibiotics resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria (Zulkifli, *et al* ,.2009). In their findings, they observed that *V. fluvialis* was resistant to chloramphenicol, streptomycin, (trimethoprim and sulfamethoxazole), ampicillin, , nalidixic acid, and concur with our recently conducted research (Okoh *et al*,. 2010).

The matter became more serious after the recent characterization of an enterotoxigenic El Tor like hemolysin in *V. fluvialis*, which represents one of the virulence factors (Kothary,*et al*, 2003). An increase in the emergence of multi-antibiotics resistant bacteria in recent years is worrisome and the presence of antibiotics resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria (Zulkifli, *et al* ,.2009). In their findings, they observed that *V. fluvialis* was resistant to chloramphenicol, streptomycin, (trimethoprim and sulfamethoxazole), ampicillin, , nalidixic acid, and, and concur with our recently conducted research (Okoh *et al*,. 2010).

This study was aim to Phenotypic characterization, antibiotic profiling, molecular characterization by16SrRNA of *V.fluvialis* species from diarrheal children.

2.Materials and methods:

2.1 Isolation of *V. fluvialis* (Elliot ,*et al*,. 2001). Enrichment media:

A total of 20 *v. fluvialis*. strains were isolated from children fecal (3 month to 11 years) in Nasiriya city were transported directally in ALkalaine pepton water (APW),PH(8.5-9.5) ,enrichement media to the laboratory and incubaction about 37 ºC at 6 hours.

2.2 Culture of samples:

After since incubation samples were spread a 10µl loop ful from incubation & cultured on Thiosulphate Citrate Bile salt Sucrose (TCBS) agar (Hi Media, India) plates by the ABC plate method and incubated at 37ºC for 24 hours. Isolated single colonies were picked, purified on nutrient agar (NA) plates ,the yellow colonies (colden),and green culture in;

1-Blood Agar : the yellow colonies (colden) cultuered on blood agar,vibrio have heamolysin &the full analysis of blood (β-heamolysis).

2- MacConkey agar: Vibrio non fermentation of lactose (yellow).

Blood agar &MacConkey agar and incubated at 37 C overnight (24 hours).

2.3 Biochemical tests(Betty,*et al*., 2007):

The results of the biochemical tests showed that to all isolated positive results for oxidase and indole ,while it gave negative results for simo-citrate,urease tests,as showen in table (4-2).

Table (4-2): result of biochemical test to *V.fluvalis*

NO.	Biochemical test	Result
1	KIA	K/A
2	H2S	_
3	Oxidase test	+
4	Indole test	+
5	Citrate test	
6	Lactose fermentation	-
7	Vp test	+
8	Ureas test	_

2.4 Genomic DNA isolation:

Genomic DNA isolation was done following the protocol of (Esteban *et al*., 1993). Used presto Tm Mini g DNA Bacteria kit (Geneaid/ uk).

2.3.2Estimation of DNA Concentration

The extracted genomic DNA is checked by using Nanodrop spectrophotometer which measures DNA concentration (ng/µl) and checks the DNA purity by reading the absorbance at (260 /280 nm).

2.3.3Molecular Characterization and 16S rRNA Gene Sequence Analysis for Identification of Species

The presumptive Vibrio isolates were subjected to molecular characterization using 16S rRNA, showed in table (2.3.3.1)Primer pairs used for identification of *V.fluvialis*,the primer sets, their corresponding gene targets and size of expected amplification products are presented figure ((Clinical Laboratory Standards Institute. 2006).

PCR was designed to target the 16S rRNA gene of *Vibrio* spp. was performed in 20 µl reaction mixture containing 4.0 µl of 5X PCR buffer, 2.4 µl 3 mM MgCL2, 0.4 µl of 0.2 mM of deoxynucleoside triphosphate mix, 1.0 µl of 0.5 µM of each primer, 0.1 µl of 0.5 U/µl of taq polymerase and 2.0 µl of DNA template. Reaction mixture for 16S rRNA gene primer was heated at 96°C for 5 min in the initial denaturation

step, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 63°C for 30 s and primer extension at 72°C for 30 s. A final extension was performed at 72°C for 7 min. PCR amplicons were electrophoresed in 1.5% agarose for 1 h at 100 V and then visualized by ethidium bromide staining and UV illumination (Clinical Laboratory Standards Institute, 2006).

Table 2.3.3: Primer pairs used for identification of *V.fluvialis.*

Nameof gene	Primer Sequences (5´-3)		Produce Size (bp)	Reference
16SrRNA	F	CGG TGA AAT GCG TAG AGA T	663	Gaber S.and Samy, 2014
	R	TTA CTA GCG ATT CCG AGT C		

2.4 Antimicrobial susceptibility test:

Antibiotic susceptibility of the isolates were determined by the disc diffusion method (Bauer *et al*., 1966). Isolates were screened for susceptibility to a panel of 10 antibiotics belonging to the various antimicrobial classes in table (2.4). Susceptibility to the selected antibiotics was determined on Mueller-Hinton agar (HiMedia, India) by the disc diffusion method as described below.

1-A single, isolated colony of the test strain was picked and transferred to 3mL of sterile physiological saline.

2-Turbidity of the cell suspension was adjusted to 0.5 McFarland standards either by adding new inoculum or physiological saline.

3-A uniform smear of the culture was made on Mueller-Hinton agar plate using a sterile cotton swab.

4-Antibiotic impregnated discs (Hi Media) were placed on to the plates, each plate holding not more than five discs and incubated for 24h at 370C.

5-Results were interpreted based on the inhibition zone around the discs as provided by the manufacturer (Hi Media).

Table (2.4): Antibiotics discs ,symbol &concentraction was used in this study

NO.	Antibiotic	Symbol	Concentration µg
1	Amipcillin	AMP	10
2	Streptomycin	S	10
3	Nalidixic acid	NA	30
4	Trimethoprim	TRI	5
5	Tetrscycline	TE	10
6	Chloramphenicol	CH	10
7	Ciproflaxin	C	5
8	Polymaxin B	PB	30
9	Doxycycline	DO	10
10	Azithromycin	AZT	30

3.Results:

3.1 Isolation and characterization of *vibrio flaviali:s:*

Atotal of 200 sample of stool from children suffering from diarrhea have been collected and tested during period from (July 2016)to (February2017.only (20)samples are given growth *v. flavialis.*

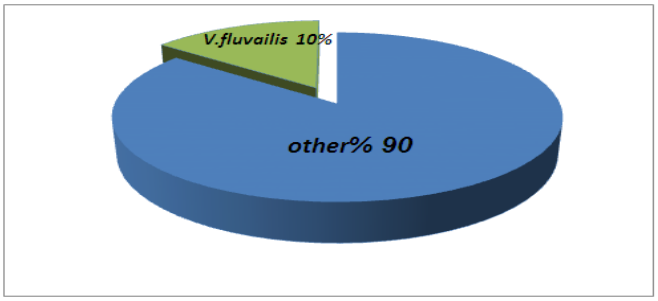

Figure (3-1): the occurrence of *Vibrio.fluvialis* isolated from 200 diarrhea children

3.2 Colony Morphology:

The results showed the different morphology characteristics of all *vibrio fluvialis* which grow on different media as in table (3.2.1) and figure (3.2.2).

Table (3.2.1): culture characteristics of *vibrio fluvialis*

NO.	Culture Media	Morphology of colonies
1	Thio sulfat citrate bile salt sucros (TCBS)	Small,rounded,smooth,yellow colonies (colden)
2	Blood Agar	small,rounded,β-haemolysis
3	MacConkey agar	Small,smooth,rounded and yellow

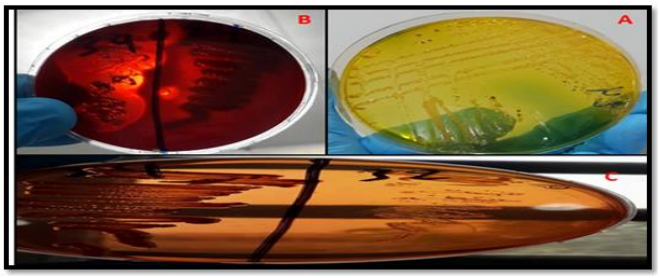

Figure (3.2.2): *vibrio* growth on different media :A - TCBS, B- Blood agar ,C - MacConkey agar

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3.3 Conventional Biochemical results:

The results of the biochemical tests showed that these isolated gave positive results for oxidase and indole ,while it gave negative results for simo-citrate,urease tests,as showen in table (3.3).

Table (3.3): biochemical test result of *V.fluvailis*

NO.	Biochemical test	Result
1	KIA	K/A
2	H2S	–
3	Oxidase test	+
4	Indole test	+
5	Citrate test	
6	Lactose fermentation	–
7	Vp test	+
8	Ureas test	–

3.4 Api-20E system identification:

Figure (3-4):Calculate the numerical profile in Api 20 system ,The test possative (code >3046126),*V.flavialis*

4.3. Molecular diagnosis of *vibrio* spp:

All the sample (20) of vibrio spp which identified by biochemical tests, API 20, and DNA extraction and PCR assay for presence of 16SrRNA gene. All the isolated was possative of that gene (100%) ,as figure (4-3).

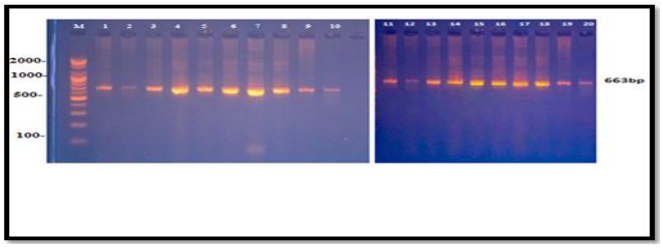

Figure (4.3): conventional PCR 16srRNA detection gene, M :Marker DNA ladder (100-1000)bp,run on 1.2%Agarose gel stained with ethidium bromide

4.4 Antimicrobial susceptibility test:

To all isolated (20)of *vibrio fluvialis* used about 10 kinds of antimicrobial agent.table (4-4-1), (4.4.2)figure (4-4-2).

Table (4.4.1): percentage of antibiotics resistance to *Vibrio fluvialis* for 10 kinds

Antimicrobial agents	NO. R	OF % R	NO. I	OF % I	NO. S	OF % S
Streptomycin	13	65%	-	-	7	35%
ciprofloxacin	9	45%	-	-	11	55%
Tetracycline	12	60%	-	-	8	40%
Chlorampheenicol	11	55%	-	-	9	45%
Ampicillin	11	55%	-	-	9	45%
Trimethoprim	10	50%	-	-	10	50%
Nalidixic acid	6	35%	1	5%	13	65%
Polymaxin B	14	70%	1	5%	6	30%
Doxycycline	12	60%	-	-	8	40%
Azithromycin	6	35%	-	-	13	65%

(R)resistance, (S)sensitive and (I)intermediate

DISCUSSION:

Vibrio fluvialis is considered to be one of the foodborne pathogenic bacteria and has been implicated in outbreaks and sporadic cases of diarrhea **(**Oliver et al ,. 2001) . Consequently, highdensities of disease causing bacteria in the watersheds are regularly reported including incidences of emerging *Vibrio fluvialis*. *Vibrio fluvialis* infection remains among those infectious diseases posing a potentially serious threat to public health. (Etinosa (O. Igbinosa and .,*et al* Anthony I. Okoh,2010).

In Serveral local in Iraq isolated *V.fluvialis* in AL Najaf Al-Ashraf Governorate (AL-Darwesh. A.ali ,2014) the study showed *V. fluvialis* strains isolated from fish also in Thi-Qar province isolated *V. fluvialis* in 2000 by (Hussien, (2000).

Data demonstrated that (20) *V.fluvialis* isolates were isolated from 300 diarrheal children with age (3 month to 11 years) and constituted about (10%), this result is disagreement with the study of (Chowdhury *et al*., 2012) which was (2%).While disagreement with (Chakraborty *et al* .,2011).

The clinical manifestation of patients from whom *V. fluvialis* was isolated as sole pathogen (54.5%) resembled that of cholera.

The reason for the difference in percentages is due to different isolation zones, environmental conditions, sinus development or the ability of bacteria to acquire new genes from other bacteria by conjugation ,transformation.

API 20E was used for confirmation of the results obtained from the biochemical tests, and the same problem was observed in the results of API 20E,. in this study result was agreement with Hussien,. (2000).in Thi-Qar province from different source.

Antimicrobial resistance has become a major medical and public health problem as it has direct link with disease management (Ramamurthy,.(2008). Antibiotics such as tetracycline, doxycycline, ciprofloxacin, streptomycin s may be used as an adjunct to rehydration therapy and are critical in the treatment of septicemia patient **(**Chiang *et al*. 2003). Polymyxin B has re-emerged in medical practice in recent years and it was resistance ,While streptomycin was resistance about (65%),and Trimethoprim its resistance about (50%) that disagreement with (Gaber &samy, 2014) was sensitive (100%) ,that resistance presence in bacteria that because transimition and conjuaction between bacterial species , while chloramphenicol was resistance about (55%) ,ampicillin (55%)that diffentioal with (Gaber & samy 2014 was While nalidixic acid was resistance at 35% that disagreement with chowdhury and Kumer (2011) was 45%. ciproflaxin was 45% it difrented with (Hussain A.(2000) was resistance at 100%.

A PCR based method using a primer pair for 16S rRNA was used for species identification of the *Vibrio* strains (Shivaji *et al*., 2000,). Since 16S rRNA gene sequence similarity of ≥97% is a reasonable level for grouping bacteria into species (Hagstrom *et al*., 2002).

By Gaber S. and Samy,2014)used 16 Sr RNA to identication *vibrio* spp The data generated using the universal 16S rRNA gene segment primers in the present study was accurate, reproducible and less time consuming compared to the conventional phenotypic identification schemes, also *V. fluvialis* strains that were identified by 16Sr RNA(Chakraborty,*et al* ,.2006). In this study was all the isolated was positive about 100% that agreement with (Gaber S and Samy l, 2014).Also agreement with(Joseph.V. Alphonsa, 2013).

Conclustions:

1-The proporation of isolation *V.fluvialis* from diarrheal children is (10%).
2- children in age (3 months-11 years were susceptible to infection with diarrhea and more susceptible for *V.fluvialis.*
3- Diagnosis of *V.fluvialis* a molecule by a gene 16SrRNA.
4- many isolated was resistance of many antimicrobial agent.

Recommendations:

1-The use of 16 SrRNA gene in most diagnosis and research laboratories is very useful for *V.fluvialis* identification techniques this method is the simplest and less expensive.
2-Studying the DNA sequencing of the PCR products for 16 SrRNA gene.
3-Futher investigations to study of *V.fluvialis* resistant gene and MIC antimicrobial resistance.
4-Study the relationship between antimicrobial resistance and virulence factors in *V.fluvialis*.

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