Identification of differences in virulence factors production from mutant isolates of clinical *Vibrio cholerae* S

تشخيص الاختلافات في انتاج عوامل الضراوة من عزلات طافرة للبكتريا المرضية *Vibrio cholerae* S

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Abstract:

Antibiotic resistant mutants for rifampicin, streptomycin and klindamycin were isolated from the clinical isolate of *Vibrio cholerae* S mutated by chemical mutagens. Mutation frequency of *V. cholerae* S depends on the treatment time and the highest viable count of antibiotic resistant were for Rifampicin after treatment with Acridine orange, Ethedium bromide, Nitrosoguanidine, 5-Florouracil, 2-Bromouracil and cyclophosphamide. One thousand mutant isolates were examined for morphological differences in colony surface, color and diameter. The treatment with AO, NTG, 5-FU, and 2-BU gave opaque to orange color larger diameter about 5-7 mm of Rifampicin resistant mutant isolates at TSA and 25% of these mutant appeared as wrinkled surface. Klindamycin resistant mutants of *V. cholerae* S were appeared as similar to the wild type while, Streptomycin resistant mutants of appeared as pin- point white smooth colonies on TSA. No differences were seen for oxidase, string test and fermentation pattern for sucrose and lactose. Toxin Coregulated Pili production was differed from high level order designated as +++ to mild ++ and low level designated as + after mutation with 5-FU and 2-BU. However, 15% of rifampicin resistant mutant isolates gave no agglutination phenomena. No proteases activity detected even after 48 hour of incubation; the production of lipases enzyme did not affected; while, mutator isolates produced high level of β-haemolysins about 2.5 fold. About 90% of cyclophosphamide- rifampicin mutant showed homogenized culture with no auto agglutination but high level of proteases. While, only 10% of cyclophosphamide - rifampicin mutants gave slightly auto agglutination and didn't produce proteases enzyme. The production of both TCP and CT were increased from rough pigment producing mutant isolates comparing with yellow and smooth mutants.

المستخلص:

تم عزل طافرات مقاومة لمضادات الرفامبسين والستربتومايسين والكلندامايسين من العزلة المرضية *Vibrio cholerae* S بعد تطفيرها بالمطفرات الكيميائية . اعتمد تكرار الطفرات على وقت التعريض وكان اكبر عدد حيتم الحصول عليه لطفرات المقاومة للريفامبسين بعد المعاملة بالمطفرات Acridine orange و

Ethedium bromide و Nitrosoguandine و Florouracil -5 و 2-Bromouracil و Cyclophosphamide . فحصت الختلافات المظهرية لالف عزلة طافرة من حيث سطح المستعمرة ولونها ومظهرها. اعطا التعريض بالمطفرات AO،5-FU/NTG،2-BU طافرات مقاومة للريفامبسين ذات لون غامق برتقالي وقطر اكبر (5 7)ميلمترعلى الوسط TSA وكان 25% من هذه الطافرات ذات سطح خشن مجعد . ظهرت الطافرات المقاومة للكلندامايسين شبهة بالمستعمرات الاصل بينما كانت المستعمرات الطافرة المقاومة للستريبتومايسين صغيرة الحجم (نقطية) بيضاء اللون ملساء السطح . لم تظهر اختلافات لفحوصات الاوكسيديز وفحص تكون خيوط الدناونمط تخمير كل من سكري اللاكتوز والسكروز . اختلف انتاج عامل الالتصاق الهدبي (TCP) من عالي الانتاج واشير له+++ لمتوسط الانتاج ++ وقليل الانتاج+ بعد التطفير بالمطفرات 2-BU، 5-FU مع ذلك لم تعط 15% من هذه الطافرات أي تلازن يذكر . لم تلاحظ فعالية لانزيم الحال للبروتين حتى بعد 48 ساعة حضن بينما لم تختلف انتاجية الانزيم الحال للدهون ولوحظ ازدياد الانزيم الحال للدم نوع بيتا لمرتين ونصف . اظهرت 90% من طافرات الريفامبسين المعاملة بالسايكلوفوسفامايد مزروع بكتيري متجانسليس به تكتل للخلايا مع انتاج عال للانزيم الحال للبروتين واعطت 15% من هذه الطافرات تكتل قليل للخلايا وانتاجية معدومة للانزيم الحال للبروتين . ازدياد انتاج عامل الالتصاق الهدبي وذيفان الكوليرا من العزلات الطافرة ذات السطح الخشن المنتجة للصبغة مقارنة مع الطفرات ذات السطح الاملس ذات اللون الاصفر .

Introduction

The study of any microorganism's properties can be greatly enhanced by the generation of mutation in genes of interest. Creation of mutations and subsequent genetic mapping can elucidate the identity, relative size, number and organization of genes involved in a physiological process. Also, the recognition and location of transcriptional units can be defined by mutation. Mutagenesis is used to create strains with desired proprieties, such as the ability to overproduce a desired metabolite or enzymes and vaccines development [1, 2]. Mutation of a gene or genes under study can be first altering the DNA of microorganisms which determines the information and characteristics of cell; however the mutation is rare in nature. It cause a problem of an investigator is trying to mass collection of a specific type of a mutation for genetic study or microorganism improvement [3]. To increase the mutation frequency three general treatments can be used to mutagenized microorganism: electromagnetic radiation, chemical mutagens, and transposons [1].

A great variety of chemical mutagens extensively used for the induction of mutations. These compounds are added to a growing culture of an organism for a given time period and interfere with replication of the DNA. Some mutagens achieves this by serving as base analogue as 2- bromouracil (2BU) and 5- florouracil (5-FU), other class can insert or intercalate in between the bases pairs of DNA causing DNA polymerase to make mistakes as ethedium bromide (Eth.Br) and acridine orange (AO). In all cases, the mutagen causes incorrect copying of the DNA resulting in base substitution, insertion, or deletion [4]. Treatment of *Escherichia coli* and classical strain 569B of *Vibrio cholerae* with nitrosoguanidine (NTG) gave mutator isolates blocked in enterotoxin production to improove attenuated live oral vaccines. Several hypotoxigenic mutants were noted to be unstable in the rabbit intestinal loop model; during passage, they produced toxigenic relevant which eventually displaced the mutant strain in vivo [5, 6]. On the other hand, cholera toxin (CT) production increased from *V. cholerae* strains treated with mitomycin C the DNA damaging agents which known to

play a role in lysogenic phages induction and to increase CTXØ titer and prophage carried out by strains[7]. This research came to study the effects of different type of chemical mutagens on the production of virulence factors from *V. cholerae* S isolate and to identify the relationship among these factors. Als,o to induce CT production at non permissive conditions in order to overcome the restriction of environment on CT production.

Materials and Methods:

Bacterial isolate:

V. cholerae S isolate was chosen for mutation experiments because its higher production of cholera toxin on production medium (0.4 of peptone, 0.5 of yeast extract, 0.4 of NaCl, 0.2 of both asparagines and glucose pH 8.5 at 35^{O}C) and neglible amount of CT production on AKI broth (0.4 of peptone, 0.5 of yeast extract and 0.4 of NaCl pH 6.9 at 37^{O}C) as determined by [8].

Preparation of culture for mutation experiments:

Overnight culture of *V. cholerae* S grown on LB broth was centrifuged at 5000 rpm for 10 minutes and bacterial pellet was washed and suspended by phosphate buffer 0.15M pH5.5. Suspended culture was incubated for 0, 15, 30, 60 minute at 37, serial dilutions were made and colony forming unit (cfu) was counted for each treatment [9].

utation of *V. cholerae* SDifferent chemical mutagens were screened for their mutagenic efficiency on *V. cholerae* S isolate under investigation. Antibiotics resistant for Rifampicin, Streptomycin and Klindamycin were used as an indicator for mutation

Antibiotic preparation

Stock solutions of Streptomysin, Rifampicin and Klindamycin were prepared at 10mg /ml, 30 mg/ml and 25 mg/ml respectively. Final concentration of 100µg/ml of streptomysin, 300µg/ml of rifampicin and 250 µg/ml of klindamycin were used for screening of antibiotic resistant mutant isolates.

Antibiotic susceptibility test:

V.cholerae S isolate was tested for rifampicin, streptomycin and klindamycin susceptibility by disc method and antibiotic incorporation method [10].

Mutation protocol

Aliquot of 20µl, 40 µl, 50 µl and 75 µl of each mutagen was added to one millileter of bacterial suspension counting 10^{8}cfu to give 40µg /ml, 80µg /ml, 100µg /ml, 150 µg /ml of mutagen respectively. Mixtures were incubated for 15 and 30 minute at 37C and serial dilutions were made to determine the killing effect of each mutagen [9]. Also, mixtures were washed tow time with normal saline and bacterial pellet was suspended with 10 ml of LB broth and incubated at 37C at low speed shaker incubator for 18 hour. Bacterial numbers and Antibiotic resistant mutations were counted for each concentration of mutagen.

Production of virulence factors from mutant isolates

One thousand mutant isolates Table (1) were screened for their differences in virulence factors production.

Table (1): The mutant isolates of *V. cholerae* S

Mutant isolates screened (CFU)	Mutagen	Antibiotic resistant
300	5-FU	Rif
100	2-BU	Rif
250	NTG	Rif
150	Acridine orange	Klin
100	Cyclophosphamide	Rif
100	Ethedium bromide	Rif

Morphological characters

Colonies shape of mutant isolates and their appearance on the surface of tryptic soya agar (TSA) were studied. Fermentation pattern of sucrose and lactose were tested on thiosulfate citrate bile salt agar (TCBS) and Macckongy agar and compared with the non mutant isolate of *V. cholerae* S.

The production of Proteases, Lipases, Haemolysins

Mutant isolates and the wild type of *V cholerae* S were streaked on TSA containing either 1% casein or 1% tween 80 or 7% sheep blood and incubated for 18-48 hour at 37°C. Cleared or turbid zone was used to detect proteases and lipases respectively. The type of blood haemolysis around bacterial growth on blood agar determined the production and type of haemolysin [8].

Detection of Toxin coregulated pili (TCP):

Vibrio cholerae hydrophobicity increased in broth culture due to the expression of pili causing visible clumping of bacteria as a pellet at the bottom of tube and leaving a clear supernatant; this phenomenon is known as autoagglutination which detected by naked eyes examination [11].

Overnight cultures of AKI broth pH 6.9 of mutant isolates were examined for Autoagglutination phenomenon. Also, cultures of production medium pH8.5 and AKI broth pH 6.9 of wild type of *V cholerae* S were used for positive and negative control respectively [8].

CT production

Only twenty out of one thousand mutant isolates were chosen and cultured in broth pH 6.9 for CT production. Also positive and negative controls were prepared from the wild type of *V cholerae* S.

Extraction, Concentration and desalting of CT:

Overnight cultures were centrifuged at 5000 rpm for 10 minutes to prepare cell free extracts which concentrated to 80% saturation of Ammonium Sulfate, and desalting of toxins protein through gel filtration by Sephadex G25 [12].

Detection methods of CT:

Qualitative detection of CT was done by the Guinea pigs permeability factor (PF). While, Toxin Units was calculated for quantitative measurement in which each 5-8 mm of erythemal activity (EA) is equivalent to 1 Toxin Unit (TU) of enterotoxin [13].

Results and Discussions

The mutation of *V. cholerae* S

The rarity of mutations is a problem if an investigator is trying to amass collection of a specific type of mutation to serve study purpose. Researchers respond to this problem in two ways, one of them is to increase the mutation rate using mutagenic agents that have the biological effect of inducing mutations above the background, or spontaneous rate.

Results reflected that no changes in *V.cholerae* S viable count occurred after 60 min incubation time on buffer pH 5.5 at 37°C (table 2). The species *V.cholerae* known to be very sensitive to low pH. Many researches recommended the use of appropriate solvent and vehicle of mutagens which should not be suspected to chemicals reactions with the mutagen and should be compatible with the survival of the bacteria [14].

Table (2): The viable count of *V. Cholerae* S isolate incubated for different period on buffer pH 5.5

Incubation time (min)	Viable count (cfu/ml)
Zero	7×10^8
15	5×10^8
30	5×10^8
60	2×10^8

Antibiotics susceptibility test revealed that growth of *V.cholerae* S inhibited by the three antibiotics. Inhibition zones reached to 20mm, 18mm and 22mm for rifampicin, streptomycin and klindamycin respectively. No viable count detected even after 72 hour incubation of TSA containing either rifampicin, streptomycin or klindamycin. These three genetic determinants responsible for the antibiotic resistant could consider as a stable genetic markers because they reside on bacterial chromosome comparing with other antibiotic determinants harbor on mobile genetics element which may lost during bacterial routine subculturing or at elevated temperatures as described by [10, 15].

Results found that the use of 40µg/ml of A.O and Eth.br and 80 µg/ml NTG led to decrease in bacterial number two logarithmic cycles, while the use of 100 µg/ml and 150 µg/ml causing four logarithmic cycles decreased with aggregation of bacteria leading to difficult correct estimation of viable bacterial number. The use of 40 µg/ml and 80 µg/ml of 5-FU and 2-BU had no effect on the logarithmic number of bacteria but at 100 µg/ml and 150 µg/ml there was a little number reduction about less than one logarithmic cycle. This result agreed with [1] when mentioned that many of chemicals are relatively non-toxic with little killing degree. The success of the mutation experiment depends on the use of low killing mutagen concentration because excessive bacterial killing by mutagen cause a decrease in mutation frequency. Also lethal mutagenesis might happen when raising the genomic mutation rate to the point that the deleterious load causes decline the viable count of microorganism. Agreed with [16] when found that mutagenesis can require many generation, allowing the bacterial population to grow to large absolute number before

the load of deleterious mutation causes the decline. On the other hand, microorganisms contain many types of DNA repair systems responsible for the correction of DNA mistakes.

Random mutagenesis by chemical mutagens is relatively simple and popular method for generating molecular diversity.

Results investigated that the highest antibiotic resistant incidence was found for Rifampicin for all mutagenic substances used. This may due to the large number of genes that responsible for RNA polymerase synthesis which consist of many subunits overlap between them. Also Rifampicin antibiotic binds to B´ subunit leading to cell death while any alter in the structure of one of these subunits led to loss the capability of rifampicin in binding and killing effect causing cell resistant [15].

Viable count of antibiotic resistant mutant cell of *V. cholerae* S under study depends on the concentration of a mutagen. Results showed that high concentration of 5-FU and 2- BU gave the higher number of Rifampicin resistant mutant isolates at contrast with, Eth.Br which induced Rifampicin resistant mutants at low concentration. While, only 500 rifampicin mutant cells/ ml were isolated after treatment with 150µg/ml of Cyclophosphamide. This may due to the need of bioactivation by cytochrom p-450 which refer to a family of enzymes that are of central importance in catalyzing the hydroxylation of a variety of organic compound and play an important role in the biosynthesis and degradation of such compound [14].

Highest viable count of Streptomycin resistant mutant of *V. cholerae* S reached to 290 mutant cell/ml when culture treated with 80µg/ml of NTG. Klindamycin resistant mutant isolates appeared from cultured mutated with low concentration of both AO and EthBr which gave 1000, and 2500 Klindamycin resistant mutant cell/ml respectively Table(3).

Table (3): The viable count of antibiotic resistant mutant after mutation

Mutagen concentration (µg/ml)	Mutagen substances		
	CFU of antibiotic resistant mutants		
	Rifampicin	Streptomycin	Klindamycin
	2-BU		
0	-	-	-
40	40	-	-
80	-	-	-
100	200	-	-
150	30	-	-
	5-FU		
0	-	-	-
40	-	-	-
80	35	30	-
100	300	55	-
150	-	-	-
			Et Br
0	-	-	-
40	200	-	-
80	35	-	-
100	40	-	-
150	50	30	-
			AO
0	-	-	-
40	-	-	100
80	-	-	-
100	-	-	-
150	40	-	-
	NTG		
0	-	-	-
40	40	150	-
80	50	290	-
100	-	-	-
150	-	-	-
	Cyclophosphamide		
0	-	-	-
40	35	-	-
80	165	-	-
100	278	-	-
150	500	13	-

The study of differences in colonies size, color and their surface showed that the treatment with AO, NTG, 5-FU, and 2-BU gave opaque to orange color of Rifampicin resistant mutant isolates at TSA Figure (1). This may resulted in melanin- like pigment production. Pigmentation is considered to be one of important microbial traits involved in resistance to antimicrobial factors and oxidative damage. The result agreed with [17] when identified the pigment producing mutant isolate of V. cholerae. Also with [18] who improved a hyper toxigenic brown- pigment producing strain of V.cholerae 569B classical Inaba by treatment with chemical mutagens.

A B

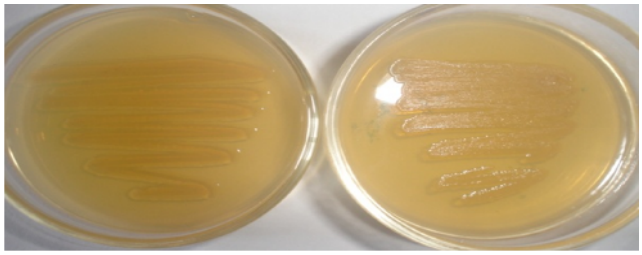


Fig (1): Growth of wild type
(A) and mutant isolate (B) of *V.cholerae* S on TSA.

Isolated colonies showed larger diameter about 5-7 mm comparing with 2-3 mm diameter of wild type of *V. cholerae* S. Only 25% of these mutant appeared as wrinkled surface as shown in figure (2). This may due to the defect in galactose metabolism pathway or/ and the flagellum biosynthesis pathway agreement with [19] who characterized and found that the galU and galE mutants gene and rough-LPS mutant comprising altered core oligosaccharide. Also, provided evidence that galU and galE are involved in rugose polysaccharide production.

Study results showed that treatment with Eth. Br and Cyclophosphamide gave Rifampcin resistant mutant isolates similar in color, size and surface to the wild type *V. cholerae* S. Klindamycin resistant mutants of *V. cholerae* S gave typical colonies regarded to their shapes, surfaces, color, and sizes on TSA. While, Streptomycin resistant mutants of *V. cholerae* S appeared as pin- point white smooth colonies on TSA.

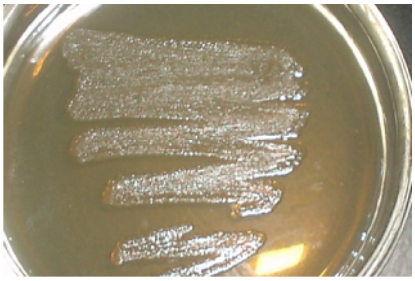


Fig (2): Wrinkled growth of the mutant isolate of *V.cholerae* FU42 on TSA

The appearance of mutant isolates similar with the wild type may resulted from the nature of the chemical structure of both A.O and Eth. Br which may needed more time for inducing another type of mutation after their intercalating between DNA strand beside this the high molecular weight of A.O made difficulties in its mutagenic action on bacterial population. While, pin point streptomycin resistant mutant may resulted from the deleterious effect of NTG which attack different reactive groups of DNA strand modify bases and/ or phosphate or oxygen by alkylating them. The DNA becomes distorted as a result and the ability of proteins to recognize and bind correctly is hindered.

Mutator isolates of Rifampcin klindamycin and streptomycin resistance were sucrose fermented, appeared as yellow colonies on TCBS agar (Figure 3). Non- Lactose fermented appeared as pale colonies on MacCongy agar. Also gave positive results with oxidase and string test.

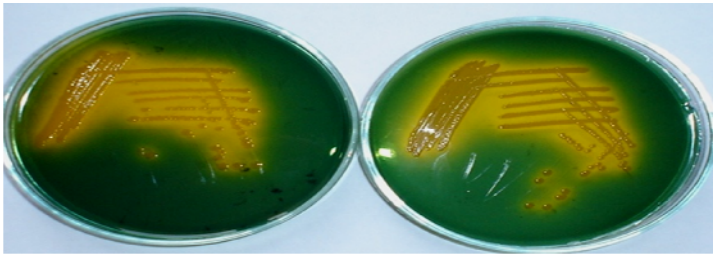
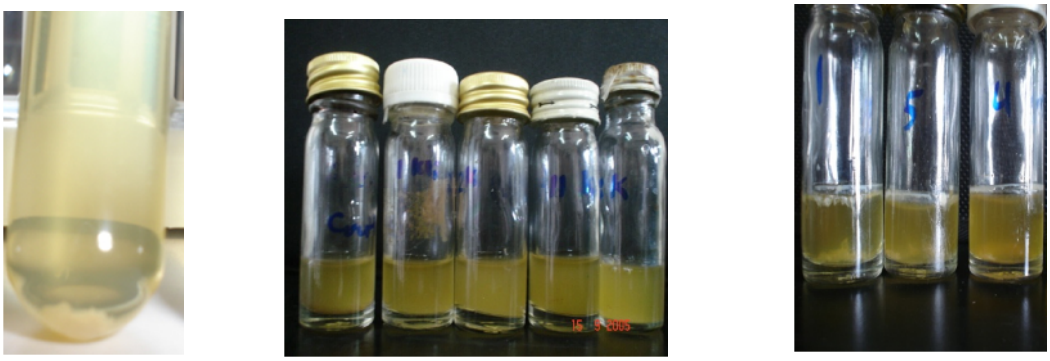


Figure (3): Growth of *V.cholerae* S on TCBS agar
(A) Wild type of *V.cholerae* S
(B) Resistant mutant isolate

Results investigated that the base analogue mutagens 5-FU and 2-BU were more efficient in the induction of mutation in *V. cholerae* S compared with the highly mutagenic substance NTG. This may due to use bacterial population at mid of stationary phase where the bacterial population did not undergo division. Further characterization and comparison in the production of TCP, proteases, lipases and haemolysin were done among 1000 out mutant isolates of *V. cholerae* S.

Results showed that the treatment with 5-FU and 2-BU gave mutant isolates differed in TCP production. Autoagglutination ranged from high level order designated as +++ to mild ++ and low level designated as + Figure (4A). However, 15% of rifampicin resistant mutant isolates gave no agglutination phenomenon as shown figure (4).



1 2 3 4 5 6 7 8 9

Figure (4): Autoagglutination phenomenon of the wild type and mutant isolates of *V.cholerae* S
1- -Positive control of the wild type of *V.cholerae* S (production medium pH8.5).
2- Negative control of the wild type of *V.cholerae* S (AKI pH6.9).
3- 4- 5- 6 Non TCP producer mutant isolate of *V.cholerae* S.
7- +++ High TCP producer mutant isolate of *V.cholerae* S
8- ++Mild TCP producer mutant isolate of *V.cholerae* S
9- Low TCP producer mutant isolate of *V.cholerae* S

No proteases activity detected even after 48 hour of incubation comparing with 21 mm diameter of cleared zone appeared around the growth of wild type. The production of lipases enzyme did not affected after treatment as show

The diameter of turbid zone to bacterial growth reached to 23mm among mutant isolates and the wild type of *V. cholerae* S.

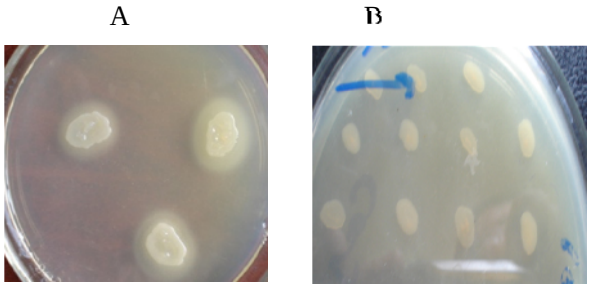


Figure (5): Lipases produced by wild type of *V. cholerae* S (A) and the mutant isolates (B).

While, mutator isolates produced high level of β- haemolysins about 2.5 fold comparing with wild type Figure (6). The diameter of hallow zone to bacterial growth of mutant isolates reached to 10:3 mm compared with 4:3mm of hallow zone detected around the wild type.

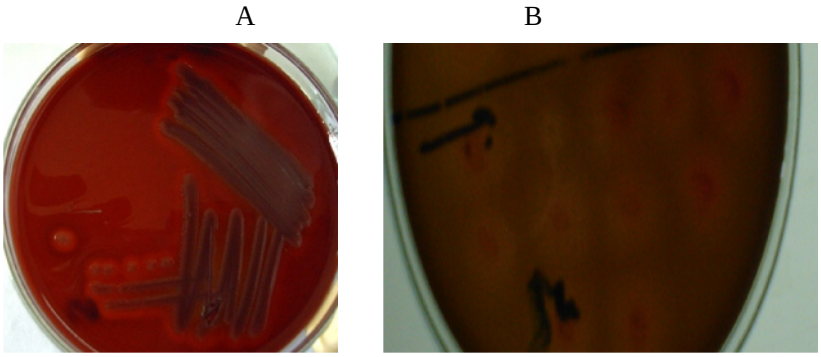


Figure (6): β - haemolysin production from the wild type of *V. cholerae* S (A) and the mutant isolates (B)

The screening of NTG and AO treated isolates revealed that only 10% of rifampicin and klindamycin resistant mutant isolates produced little TCP, proteases couldn't detected even after 48 hour incubation, ordinary level of lipases and haemolysins enzymes.

About 90% of cyclophosphamide- rifampicin mutant showed homogenized culture with no autoagglutination but high level of proteases about two fold increments were detected. While, only 10% of cyclophosphamide - rifampicin mutants gave slightly autoagglutination appeared at the bottom of the tube and didn't produce proteases enzyme. These entire mutants produced ordinary amount of lipases and haemolysines compared with wild type. Ethedium bromide treatment gave mutations similar with the wild type in regarded to autoagglutination, proteases, lipases and haemolysins enzymes production.

Results for twenty different mutant isolates choosing from different treatments indicated that both TCP and CT production increased from rough pigment producing mutant isolates 42FU, 2FU, 3FU, 49FU, 135 FU, 14N and 13N comparing with

yellow and smooth mutants. The appearance of rough opaque colonies with high TCP production may result in the increased pathogenicity and virulence of *V.cholerae* S for its host. Also, the existence of a correlation between production level of TCP, proteases enzyme and CT production. There were three levels of TCP and proteases production. The neiglable quantity of proteases connected with high level of autoagglutination as shown in Table (4).

Table (4) Comparison of virulence factors production among twenty mutant isolates and the wild type *V. cholerae* S

Bacterial isolate	Mutagen	Resistant marker	color of colony	surface of colony	Protease	TCP	EA (mm)	Toxin unit/ ml
42FU	5-FU	Rifampicin	Opaque	Wrinkle	-	+++	13	26
2FU	5-FU	Rifampicin	Opaque	Wrinkle	-	+++	12	24
3FU	5-FU	Rifampicin	Opaque	Wrinkle	-	+++	12	24
4FU	5-FU	Rifampicin	Yellow	Smooth	+	-	-	-
17FU	5-FU	Rifampicin	Yellow	Smooth	+	-	-	-
21FU	5-FU	Rifampicin	Opaque	Smooth	-	++	10	20
24FU	5-FU	Rifampicin	Yellow	Smooth	delayed	+	5	10
30FU	5-FU	Rifampicin	Yellow	Smooth	-	++	10	20
35FU	5-FU	Rifampicin	Yellow	Smooth	delayed	+	7	14
36FU	5-FU	Rifampicin	Yellow	Smooth	-	+	6	12
39FU	5-FU	Rifampicin	Yellow	Smooth	+	-	-	-
50FU	5-FU	Rifampicin	Yellow	Smooth	+	-	-	-
85FU	5-FU	Rifampicin	Yellow	Smooth	+	-	-	-24
94FU	5-FU	Rifampicin	Opaque	Wrinkle	-	+++	12	24
135FU	5-FU	Rifampicin	Opaque	Wrinkle	-	+++	12	20
13N	NTG	Rifampicin	Opaque	Smooth	-	++	10	20
14N	NTG	Rifampicin	Opaque	Smooth	-	++	++	-
1KA	AO	Klindamycin	Yellow	Smooth	-	-	-	20
13KA	AO	Klindamycin	Yellow	Smooth	-	++	10	-
45ET	EthBr	Klindamycin	Yellow	Smooth	+	-	-	
Wild type S**	-	-	Yellow	Smooth	+	++++	17	34
Wild type S*	-	-	Yellow	Smooth	+	-	-	-

-= negative; +=positive; *= negative control (AKI pH6.9 and 37 °C)
****= positive control (production medium pH 8.5 and 35°C)**

The appearance of mutant isolates of *V. cholerae* S lacking protease activity may due to the mutation either in the structural gene *hap*A gene which affected the production of enzyme or in the secretion pathway of proteases which composed of many different genes that affected and dysfunction by mutation. Agreement with (20) who determined a little proteases producing isolate of vibrios which lacking a functional *hap* A gene.

The mutant isolates of *V. cholerae* S lacking proteases activity or high level proteases production at non permissive conditions might have a defect at one or more of regulatory proteins. On the other hand, quorum sensing regulators are involved in regulation of *V. cholerae* virulence genes in response to cell density. Both [20] and [21] suggested that the expression of a subset of virulence factors of *V.cholerae* is coordinately controlled by a regulatory cascade. Also, demonstrated that the well

characterized ToxR signal transduction cascade responsible for sensing and integrating the environmental information and controlling the virulence regulon. Many of the *tox*R activated genes involved in the production of TCP and CT. While, the mutant at *tox*R showed the dramatic transcriptional changes compared with the wild type. A total of 154 genes showed 1/2 fold change in transcription, 60 genes showed increased expression in the mutant strain and 94 genes showed decreased expression in the mutant strain. With the same respect, [20] confirmed the role of LuxO the quorum sensing regulator as a central "switch" that coordinately regulates virulence –related phenotype such as proteases.

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