The Effect of Coumarin Derivatives(compounds) on the *Vibrio cholerae* Isolates from Different Clinical Iraqi Sources

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Abstract

From a large number of bacterial samples collected from different hospital in Iraq in central health laboratory ,only ten isolates were identified primary as *Vibrio*. A number of morphology and biochemical test were carried out to complete this identification that showed all bacterial isolates were related to *Vibrio cholerae* .In this study all *Vibrio* isolates were investigated for Bio typing and the result showed that all (10) isolate were related to (Eltor biotypes) .Also, the susceptibility test towards eight antibiotics were carried out .

Results shows that ciprofloxacin , Norfloxacin, Erythromycin, Ampicillin, ceftriaxone and Amikacin were the most effective antibiotics and their resistance percentage were 20%,20%,20, 20,30% and 30% respectively ,While Chloramphenicol and Co- trimoxazole were less effective and their resistance percentage were 90% both of them. Three (3,5,6) isolates *V. cholerae* were selected depending on results of antibiotics sensitivity tests as showed multiple –antibiotics resistance(100%).

Then tested to study the effect of coumarin derivatives compounds (1, 2, 3) which showed inhibitory effect on *V. cholera* (3,5,6) isolates and the compound (3) showed the highest antibacterial activity of (12,15,14 mm) of inhibition zone diameter against *V. cholera* (3,5,6) isolates respectively.

Also, these Iraqi isolates (3,5,6) used to test the effect of acridine orange (0.1%) as acuring agent , the results showed that all (3) isolates *V. cholerae* were sensitive to (ciprofloxacin, ceftrixone and Norfloxacin), While the rest were resistance to remained five antibiotics.

The results of Agarose –gel electrophoresis of both normal *V. cholerae* (3,5,6) and cured isolates showed the presence of chromosomal and plasmid DNA bands in the normal case ,While only chromosomal DNA bands occur with *V. cholerae* (isolate 8) treated with an acridine orange at concentration of (10^{-2} to 10^{-4}).

Keywords: ***Vibrio cholera,*** **Coumarin derivatives, Acridine orange.**

تأثير مركبات الكومارين على عزلات من بكتريا *Vibrio cholerae* المعزولة من مصادر سريرية عراقية مختلفة

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الخلاصة

من مجموع عدد كبير من نماذج بكتيرية جمعت في مختبر الصحة المركزي من مستشفيات مختلفة في العراق للفترة من ٢٠١٥/١١/١- ٢٠١٦/٤/١ ,تم الحصول على (١٠) عزلات شخصت مبدئيا على انها تعود الى جنس *vibrio* .تم اجراء عدد من الفحوصات المظهرية والكيموحياتية لا كمال هذا التشخيص مما اظهر ان هذه العزلات تعود الى *Vibrio cholerae*.

شملت هذه الدراسة اخضاع هذه العزلات الى التنميط الحيوي ((Bio typing وقد اظهرت النتائج ان جميع هذه العزلات العشرة كانت من نوع (Eltor).كما تم قياس حساسية هذه العزلات تجاه ثمانية (٨) من المضادات الحيوية , واظهرت النتائج ان السبروفلوكساسين , نورفلوكساسين ,الارثرومايسين, الامبسلين والسيفرتوكسين والاميكاسين كانت اكثرالمضادات تاثيرا وبنسبة مقاومة تساوي ٢٠%,٢٠%,٢٠%,٢٠%,30%,30% على التوالي, بينما كانت للكلورمفنيكول والكواترامكسول اقل تاثيرا حيث كانت نسبة المقاومة ٩٠% لكليهما. تم اختبار ثلاث عزلات من بكتريا*V. cholerae* هي (٦,٥,٣) اعتمادا على نتائج الحساسية للمضادات الحيوية والتي اظهرت مقاومة متعددة بنسبة (١٠٠%) . كما تم اخضاعها (العزلات الثلاثة) لدراسة تأثير مركبات الكومارين الثلاثة (٣,٢,١) (كلوريد الكوبلت, كلوريد المنغنيز, المركب الثالث هو مزيج من كليهما) على العزلات الثلاثة حيث اظهرت النتائج ان المركب (٣) كان له اعلى تاثير ضد بكتيري بقطر تثبيط (١٤,١٥,١٢ ملم) ضد عزلات*V. cholerae* (٦,٥,٣) على التوالي.

كما شملت هذه الدراسة اختبار حساسية المضادات من خلال دراسة تأثير مادة الاكردين البرتقالي بتركيز((0.1% كعامل محيد على هذه العزلات اعلاه وقد اظهرت النتائج ان جميع العزلات الثلاثة من البكتريا كانت حساسة للمضادات (سبروفلوكساسين ,سيفوتوكسيم والنورفلوكساسين) بينما بقية العزلات كانت مقاومة المضادات الخمسة الباقية.

اظهرت نتائج الجانب الوراثي للترحيل الكهربائي لكل من عزلات *V. cholerae* (٦,٥,٣) الطبيعية والمحيدة وجود حزمة للبلازميد في العزلات الطبيعية بينما ظهور حزمة للكروموسوم فقط لعزلات ضمات الكوليرا (العزلة ٨)المعاملة مع الاكردين البرتقالي بتركيز ($١٠^{-٤}$ الى $١٠^{-٢}$) .

الكلمات المفتاحية : بكتريا *Vibrio cholera*، مركبات الكومارين ، الاكردين البرتقالي .

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Introduction

Cholera as a disease is an acute diarrheal illness caused by *V. cholerae* infection of intestine,by how may infect 3-5 million person and may cause the death to 100,000 to 120,000 for each year ,if don't have the therapy[1]. *V.choleraee* is a Gram negative, curved rod bacterium, colonize the small intestine ,naturally habitat abrakish or salt water[2]. When ingested, *V. cholerae* can cause diarrhea and vomiting in the host within several hours to 2-3 days of ingestion [3,4].

The modern genetic studies that are achieved on the cholerae bacteria (Eltor biotype) showed that it contains two circular chromosomes instead of one [5]. One of the two chromosomes has large size, it consists of 3 million base pair, it contains the most necessary genes to produce cytotoxin CT as well as the proteins which enter in metabolic pathways for the cell [6], as well as it includes the important genes to DNA replication, the cell division, genes transcription,protein translation and building the cell well as well as the genes that encoded about the virulence factors such as toxin corregulated pili (*tcpA*) and (*toxR*)[7].

Heinemann *et al* referred to the existence type of plasmids is called R – plasmid which is existed in genetic content of the cholerae bacteria, this factor is responsible for bacteria resistance for many antibiotics [8].The transferring of R-plasmid is easy where it is transferred to bacteria (*E. coli* – K12) to study the effect on the bacteria which is transferred to it in terms of resistance and sensitivity to the antibiotics [9].

V. choleraee bacterial was sensitive to a large number of antibiotics until the seventh decade of the twentieth century, However, the widespread and non programmable using of antibiotics leads to the emergence of resistant strains to many antibiotics [10]. Until 1970 ,*V. choleraee* was sensitive to each of antibiotics(Tetracyclin,Ampicillin,Chloramphenicol), and in the eighties, It emerged resistant isolates to many antibiotics such as Ampicillin, Trimethoprime andTetracycline [11].

The coumarin compound afragrant organic chemical class (warfarin) benzopyrone, which is colorless crystalline substance in standard state , it it's a natural substance found in many plants on their biosynthesis in plants occur via hydroxylation, glycolysis and cyclization of cinnamic acid. [12, 13]

The coumarin compounds have important biological applications, it has been used as antifungal agents and as anticoagulants , and also to organize the growth of plants [14,15].

Acridine orange (Ao) is an organic compound used in nucleic acid selective fluorescent cationic dye useful for cell cycle determination at502nm and an emission maximum shifts to460nm (blue). AOwill also enter acidic compartments such aslysosomes and become pronoated and sequestered in theselow pH conditions ,the dye will emit orange light .Thus Acridine Orange can be used to identify engulfed apoptotic cells,because it will fluoresce upon engulfment .The dye is oftenused in epi florescence microscopy and flow cytometry analysis of cellular physiology and cell cycle status [15] .

Materials and Method

Samples collection

Ten bacterial samples used in this study were obtained from central healthy laboratory collected from different hospital in Iraq.

Bacterial diagnosis

Initial diagnostic depending on Gram reaction and morphological characteristic of the colonies based on bacterial growth on thiosulphate citrate bile salt sucrose agar (TCBS) and Blood agar, as well as the number of biochemical test [16],and VITEK 2 Compact system. The second technique Biotyping of bacterial ,the biotype of *V. cholerae* serogroup O1(EItor) was distinguished by the following methods; polymyxinB susceptibility ,heamolysin test and voges-proskaur test [17].

Antibiotic susceptibility test

Disk agar diffusion according to Kirby Baur standardized antimicrobial susceptibility single disk method was carried out toward eight antibiotics (Ciprofloxacin , Norfloxacin, Erythromycin, Ampicillin, ceftriaxone , Amikacin, Chloramphenicol andCo-trimoxazole), Bioanaylyse(Turkey).

The antibiotic resistance percentage detected to each antibiotic as = number of resistance isolate to a specific antibiotic /Total number of tested isolates ×100% [18] .

Experimental reparation of ethyl – 6 – chloro – 1 H – isochromene – 3- carboxylate (S)

In this study three already synthesized coumarin derivatives were used compound (1) ($Co(C_{10}H_5ClO_4)_2Cl_2$), compound (2) ($Mn(C_{10}H_5ClO_4)_2Cl_2$) and compound (3) mix of (1) and (2) compounds, and their melting point were detected (71˚C = 344F) and prepared as follow:

5-chloro–2-hydroxyl benzaldehyde(0.56 gm , 0.0036 mole) and diethyl malonate (0.64 gm , 0.004 mole 10 mol % excess) were heated with stirring in ethanol (95 % , 20 ml) until dissolution occurred . Addition of piperidine (orang . the solution was refluxed

for 6 hour and on cooling a white crystalline solid formed , crystals of ethyl – 6-chloro – 1 H – isochromene – 3-carboxylate were isolated by filtration and washed form ethanol , filtered (0˚C) , the solid was recrystallized from ethanol , filtered and washed with cold ethanol again.

An ethanolic Solution (10ml) of the metal salt (metal (II) salts are hydrated chloride ; MCl2 .XH2O ; where : M=, Mn (II) , co (II) (: X= 6, 6 ,4 and 2, respectively) , was add to methanolic solution of the ligand (2mmole) in methanol (15ml).

The reaction mixture was then refluxed around 2 hr. at PH= (6-7) , a colored precipitate was formed the product was then filtered and re-crystallized with methanol and dried at room temperature [19] .

Antimicrobial activity of the coumarin derivatives compounds

The agar-well diffusion method was used to evaluate the antimicrobial activity of the (6 – Chloro – 2 – oxo – 2 Hchromene – 3 – carboxylic acid and complexes) compounds . A bacterial suspension of ($1.5x10^6$ cells/ ml) which obtained by transfer aloopful of bacterial suspension from serial dilutions (stosk,10^{-1} , …… 10^{-6}) , then streaking on (NA) nutrient agar plates and give acolony number (150 colony / 100ml medium)/dilution) was inoculated on Muller-Hinton agar plates. Wells (5 mm in diameter) were made in solidified agar using a sterile end of Pasteur pipette. An amount of 0.1 ml($2*10^{-3}$M) of each compounds was then added to the wells, in addition, one well was filled with DMSO solution(aspecific soluble solution) , as a control (19). The plates were put in refrigerator at 4˚C for 30 min to allow for diffusion of concentrations via the medium ,then incubated for 24 hrs at 37˚C and the antimicrobial activity of each compounds was recorded by measuring the inhibition zone around the wells.

Effect of curing agent (acridine orange).

In this part of study the highly *V. choleraee* resistant isolate were used for studying the effect of the curing agent acriding orange concentrations of ($0,10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-10}$)by dissolving 0.1mg of acridine orange in 10ml D.W.then the above concentrations were prepared and added to a plate of nutrient agar seeded with alawn of nutrient broth (18-24h.) culture of isolates (3and 5). Then ,the antibiotic susceptibility were measured at each concentration [20].

DNA profile by gel electrophoresis

Bacterial plasmid DNA was extracted from cultured cells using the alkaline SDS method with promega DNA kit described by [21] as follow:

Centrifuge 25 ml of culture, resuspended pellet in 0.5ml of lysozyme solution containing(0.3M NaOH ,2% SDS), mix and incubated at 70˚C for 15min, add 0.08ml of acid phenol/chloroform (1:1)and mix gently, separate phases by centrifugation at 10000xg for 10min and transfer the upper aquous phase to a new Eppendorff tube containing 0.07M sodium acetate and 0.7ml isopropanol, centrifuge again for2min , the pellet dissolved in 0.05ml TE buffer, stored at 4˚C and samples were electrophorated using 0.7% agarose with 5v/cm for 2h, after ending of electrophoresis process,the gel was exposed to U.V. light with 340nm to observe plasmid bands.

Results and Discussion

All bacterial samples expected to be a *V. choleraee* grown on alkaline peptone water (APW) at pH 9.2 as enrichment media, then transferred to thiosulfate citrate bile salt sucrose agar medium (TCBS) and occurred as yellow brilliant colonies on this medium figure (1).Also , it occurs as comma to S-shaped – with red color under the microscope .Then ,a number of biochemical test were carried out and the results showed in Table(1).

Table (1): Biochemical tests of *V. choleraee* isolates .

Biochemical Test	***Results***
Oxidase	+
Catalase	+
Kliger Iron Assay	A/K No gas, No H2S
Mannose Resistance	+
Voges Proskaur	+
Motility	+
Urease	+
Cholerae red	+

All the above results in addition to identification by Vitek system ,revealed that all ten *Vibrio* isolates were related the cholerae species (100%)(15).

In this study ,all (10) *V. cholerae* isolates were investigated biotyping and the results showed that all *V. choleraee* were O1(Eltor) serotype group(16).Our results was in agreement with the results obtained by [22].

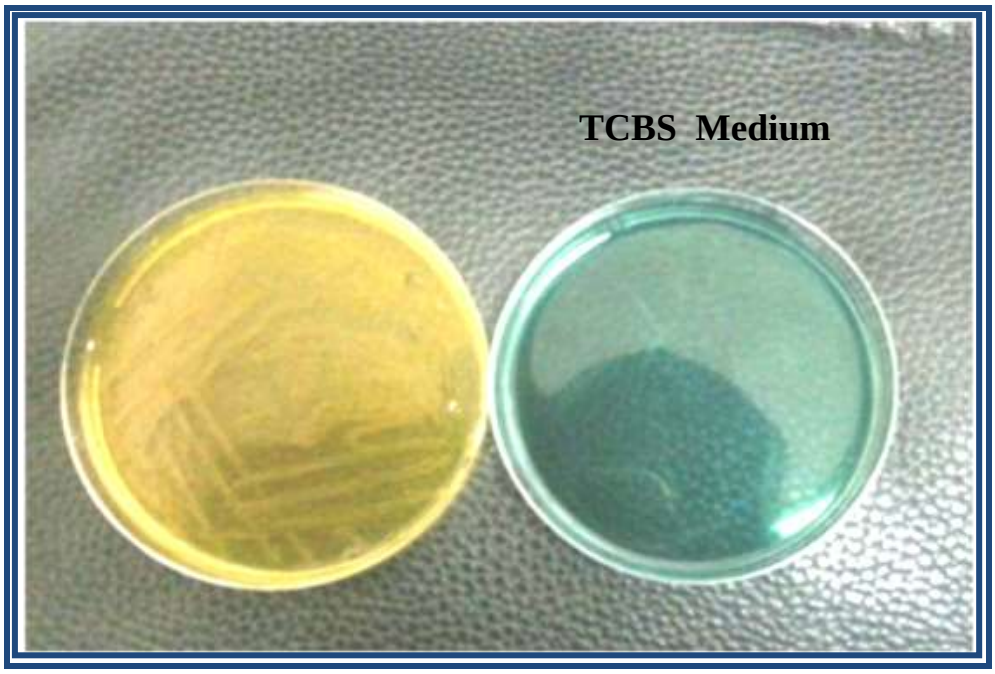

Figure (1): Thiosulfate Citrate bile salt sucrose agar (TCBS) medium for cultivation of *V. cholerae* produce a distinctive yellow colonies.

Antibiotics Susceptibility

The Susceptibility tests toward eight antibiotics were carried out and results are illustrated in figure (2) showed that ciprofloxacin , Norfloxacin,Erythromycin, Ampicillin, ceftriaxone and Amikacin were the most effective antibiotics and their resistance percentage were 20%,20%, 20,30% and 30% respectively ,While Chloramphenicol and Co- trimoxazole were less effective and their resistance percentage were 90% both of them. Three isolates *V. cholerae* (3,5,6)were selected depending on results of antibiotics sensitivity tests as showed multiple –antibiotics resistance(100%).

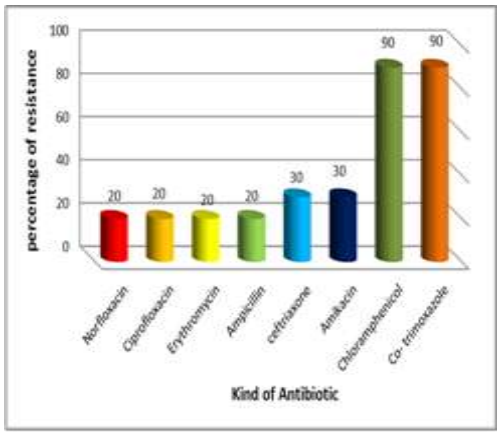

Figure (2): The resistance percentage of *V. choleraee* isolates to antibiotics.

Results agreed with [22] who found that the *V. choleraee* isolates showed a high sensitivity to each of Ampicillin**,** Ciprofloxacin, Erythromycine , Tetracycline, Gentamicin and Cephalothin, and showed a low resistance (10%) and (20%) to Cefotaxime and Amikacin respectively, but disagreed with her results when she found that (80%) of the environmental isolates were resistance to Amikacin.

The antimicrobial activityof coumarin derivatives(compounds) compounds against V. choleraee.

The antimicrobial activity of three types of coumarin derivatives(compounds 1,2,3) against three high resistance *V. choleraee*(3,5,6) were studied and results shown in the table(2) at which the compound 3 show the highest effect at which the diameter of inhibition zone was (12,15 and 14 mm) against *V. choleraee* isolates,3,5,6 respectively ,While the compound 1 was have a moderately effect at which the diameter of inhibition zone was (10,12 and 12 mm) . While the compound 2 was lowest effective by producing the inhibition zone of (0,11 and 10 mm) against the above isolates respectively.

Our results was in agreement with Coixia *etal*, 2014 who found that many compounds containing chromene ring moiety display broad spectrum of biological activity. 2H-Chromenes have gained much attention

because of various biological activities such as antiviral, anti-tumor, anti-bacterial, fungicidal, antiflamatory, antioxidative and activator of potassium channels effects[22].Coumarin (2H-1-benzopyran-2-one), a naturally occurring plant constituent, has been used in the treatment of cancer and edema, and many of its derivatives have also shown biological activity. include antibacterial [23].

Table (2): Antimicrobial activity of the coumarim compounds against *Vibrio cholera* isolates.

compounds / Bacteria isolation	(Co(C10H5 ClO4)2 Cl2) Compound (1)	(Mn(C10H5 ClO4)2Cl2) Compound (2)	(Co(C10H5 ClO4)2 Cl2) And (Mn(C10H5 ClO4)2Cl2) Compound (3)
Inhibition zone in mm			
Isolation no 3	**10**	**0**	**12**
Isolation no 5	**12**	**11**	**15**
Isolation no 6	**12**	**10**	**14**

The effect of acridine orange concentrations on Antibiotic sensitivity of three highly resistance V. cholerae isolates.

The results of the effect of acridine orange on athree higher resistance *V. cholerae* (3,5,6) were studied and shown as in table (3). These results showed that three *V. choleraee* isolates became sensitive to Ciprofloxacin, Norfloxacin and ceftrixone till 10^{-6} concentration of acridine orange While still resistance to the remained five antibiotics under this study.

Also, the results of agarose electrophoresis were shown in figure3 revealed the presence of plasmid DNA bands of (472, 670, 520 & 950)bp in (3,5,6,7) isolates respectively and chromosomal band in normal cases, while the presence of one chromosomal band isolate 8 which treated with acridine orange at concentration of (10^{-2} to 10^{-4}). The effect of keylating agent (acridine orange) may cause a genetic mutations due to the changes in antibiotics resistance types of curing isolates after one hour of electrophoresis

Table (3): The effect of acridine orange concentrations on Antibiotic sensitivity of three highly resistance *V. choleraee* isolates(3,5,6) .

Antibiotic / acridine orange con.	Cip.	Nor.	Ery.	Amp.	Ceft.	Amk.	Chlora.	Co-tri.
0	RRR	RRR	RRR	RRR	RRR	RRR	RRR	RRR
10^{-1}	SSS	SSS	RRR	RRR	SSS	RRR	RRR	RRR
10^{-2}	SSS	SSS	RRR	RRR	SSS	RRR	RRR	RRR
10^{-3}	SSS	SSS	RRR	RRR	SSS	RRR	RRR	RRR
10^{-4}	SSS	SSS	RRR	RRR	SSS	RRR	RRR	RRR
10^{-5}	SSS	SSS	RRR	RRR	SSS	RRR	RRR	RRR
10^{-6}	SSS	SSS	RRR	RRR	SSS	RRR	RRR	RRR
10^{-7}	RRR	RRR	RRR	RRR	RRR	RRR	RRR	RRR
10^{-8}	RRR	RRR	RRR	RRR	RRR	RRR	RRR	RRR
10^{-9}	RRR	RRR	RRR	RRR	RRR	RRR	RRR	RRR
10^{-10}	RRR	RRR	RRR	RRR	RRR	RRR	RRR	RRR

These results were in agreement with Hopwood *etal*, 1995 and pang *etal* 2007 who study the role of many antibiotic resistance properties of different serotypes of *V.cholera* which were plasmid determined and affected by many mutagenic agents [24,25].

Our results were in contrast with Pavlov etal 2006 who found that genetics of actinorhodine –production by *Streptomyces*

coelicolor was chromosomally determined and curing isolates treated with Ethidium-Bromide still actinorhodine producers. [26].Another study showed the presence of only one mega plasmid band of *Bacillus thuringensis* after 1:30h of electrophoresis while, there is a complex plasmid profile with different molecular weights occur within the same species. This reason may related to the differences of plasmid properties between strains related to the same species or even sub species level [27].

Bora *etal* 1994 found that these differences in plasmid profile may caused by the differences in the method of DNA separation, the molecular weights of plasmid and its copy number found in bacterial cells [28].Our results may help to investigate the effect of coumarin derivative on another pathogenic bacteria even anti-tumor activity, also it was important to determined the specific gene sequence of cured isolate and the effect of acridine orange on other virulence factors of more epidemiological agents in Iraq.

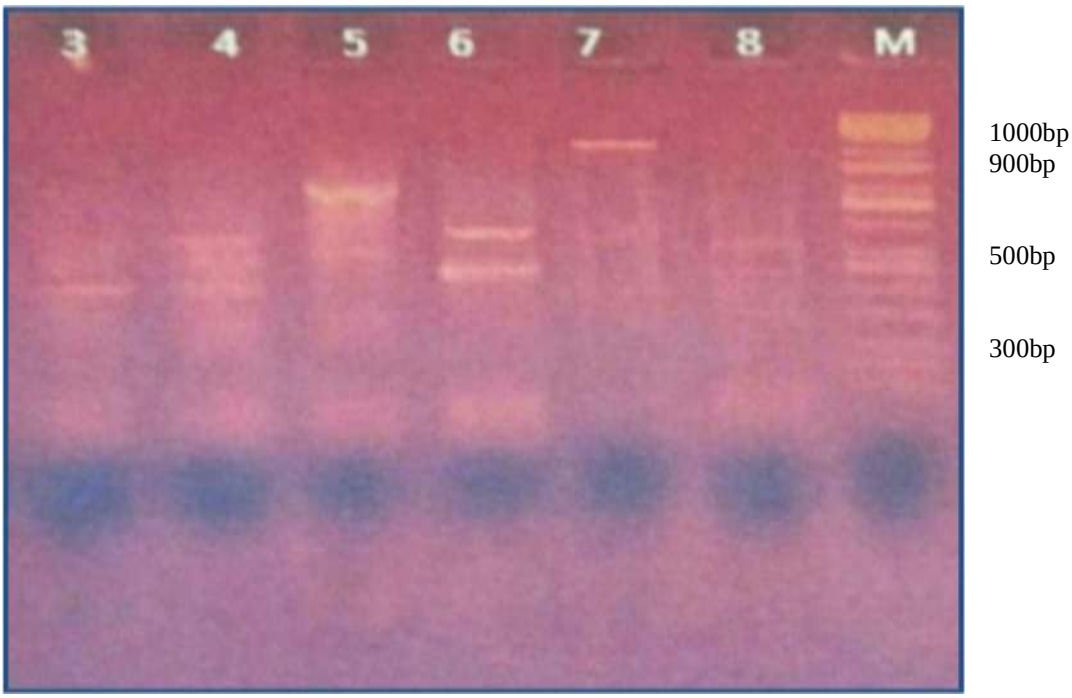

Figure (3): Agarose gel electrophoresis of both chromosomal and plasmid DNA isolated from *V. choleraee* Iraqi isolates. Lane 3,5,6,7 represent the normal isolates; lane 4 represents repeated normal isolate (3). Lane 8 represents treated isolate with Acridine orange at concentration (10^{-2}); lane M represents a marker.

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