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PREVELANCE OF VIRULENT GRAM POSITIVE BACILLI CAUSING EYE INFECTION

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Abstract

Purpose: to determine The virulence of Gram Positive Bacilli causing eyes infection .

Methods: *Bacillus cereus* was isolated from patients with bacterial conjunctivitis from April to December 2015, on MYP agar media. Fifty - four bacterial samples isolated from infected eyes. The bacterial isolates were subjected to biochemical tests ,antobiotics sensitivity and then identified by using Polymerase chain reaction (PCR). Genes; Chaperonin *Gro-EL*, Hemolysin BL(*hbl*) and Phosphatidylcholine-specific phospholipase C *(Pc-plc*) were studied.

Results: In this study out of 54 isolates, 14 have positive growth on MYP agar media. Antibiotic susceptibility test revealed that the tested isolates were highly susceptible to imipenem, ciprofloxacin and amikacin with susceptibility percentages (100 %) , followed by Vancomycin and Penicillin G with 88% and 66 % susceptibility, respectively. The isolate No 54 was selected as The most drug resistant bacteria , Then identified molecularly by amplification of Chaperonin *Gro-EL* gene .The virulence genes of *Bacillus cereus* E54; *hbl* and *Pc-plc* were studied . The genomic sequence of these genes ; *Gro-EL* , *hbl* and *Pc-plc* were submitted to GenBank at NCBI web site (www.ncbi.nlm.nih.gov) in accession number MH837160 , MH844524 and MH837161, respectively.

Conclusion: *Bacillus cereus* E54 causes eye infection was exhibited high resistance against most tested antibiotics and gave positive results with *Gro-EL*, *hbl* and *PC-plc* genes.

Key words: Bacterial conjunctivitis, Antibiotics sensitivity, *Bacillus cereus*, Chaperonin *Gro-EL* gene, Hemolysin BL(*HBL*) gene, Phosphatidylcholine-specific phospholipase C(*Pc-plc*) gene .

Introduction

Conjunctivitis refers to any inflammatory condition of the conjunctival membrane [1].It is generally characterized by irritation, itching, foreign body sensation, and watering or discharge, dilatation of the conjunctival vessels, resulting in hyperemia and edema of the conjunctiva [2], [3] It is commonly referred to as "red eye" or "pink eye [4] .

Bacterial conjunctivitis is a relatively common infection and affects all people, although a higher incidence is seen in infants, school children and the elderly. Bacterial conjunctivitis has a higher prevalence in children, where a recent study done by Rose, 2005 identified 67% of 326 children as having a bacterial conjunctivitis. Although its incidence is continuing to decrease in developing nations, periodic rises in incidence are seen during the monsoon seasons in many countries such as Bangladesh [5].

Bacterial conjunctivitis is the most common type of infective conjunctivitis in developing nations. In the United States it was estimated that 23% of bacterial conjunctivitis cases occur in the 0-2 year age range, 28% occur in the 3-9 year range, 13% occur in the 10-19 year range with the remaining 36% of cases occurring in adults [6]. In Norway, it was estimated that the prevalence of the most severe form of acute infective bacterial conjunctivitis is on the order of 30 out of 1000 patients in a general medical practice [7]. Similar clinic-based data from the United Kingdom have pointed to a rise in the proportion of patients who have sought medical attention for conjunctivitis, rising from 284 per 10,000 in 1981-1982 to 395 per 10,000 over the period 1991-1992 [6]. It has further been estimated that acute bacterial conjunctivitis represents up to 1% of all visits to general practitioners in the United Kingdom . However, it has also been noted that general practitioners tend to over-diagnosis bacterial conjunctivitis [6]. Approximately 1% of all patient visits to primary care clinics are conjunctivitis related, and the estimated cost of the bacterial conjunctivitis alone is 377 million to 857 million annually. Bacterial pathogens are isolated in only 50% of cases of suspected conjunctivitis [4].

Children younger than two years are the most commonly infected. *Haemophilus influenzae* was found to be the most common causative agent cultured from the conjunctival swabs from children but was absent from the conjunctival swabs taken from adults. The most prevalent organism from the adult group was *Staphylococcus aureus. Streptococcus pneumonia* was a

common infective agent featuring in 22.2% of the specimens from children and in 20% of the adult specimens. *Streptococcus pneumoniae* was also found to be one of the most predominant organisms found in mixed bacterial cultures along with *Haemophilus influenza* . Most cases of acute bacterial conjunctivitis occur during the winter months, which could be due to the fact that *Haemophilus influenzae* and *Streptococcus pneumoniae* infections are often a result of the migration of the bacteria from the upper respiratory tract causing secondary infections[8].The incidence of infant conjunctivitis vary geographically due to the differences in the prevalence of maternal infection and the use of prophylaxis. In US and Europe the incidence has been reported 1-2% depending on the socioeconomic character of the area [5]. In recent studies in Pakistan the incidence has been 17% and in Kenya as high 23% [5].

Therefore, the aim of this study is to investigate the ability of *Bacillus cereus* to cause eyes infection . Also to evaluate the virulence factors of the *Bacillus cereus* and effect antibiotics on these isolates. Then determining the most efficient antibiotics against the isolated *Bacillus cereus*.

Methods

Collection sample: Fifty four medical samples of bacterial ulcer were collected from different patients eyes from Sidnawy Hospital in the period from April to December 2015. The specimens were collected and transported according to Murray [9] under aseptic conditions quickly to the Microbiology Laboratory where the study was carried out.

Isolation and purification of bacterial isolates : Samples were handled by sterile clean swab and transferred to laboratory within few hours. Samples were streaked on MYP agar media to obtain single colony. Plates were incubated aerobically at 37°C for 24 h. The purified isolates were then maintained on slants of nutrient agar medium. All the slant cultures were stored in refrigerator with regular transfer every month.

Antibiotic susceptibility test: Nineteen of different antibiotics were selected for carrying out the antimicrobial susceptibility test . One ml of culture in nutrient broth was spreaded on the surface of Muller-Hinton agar media and incubated for one hour before antibiotic discs were placed on the surface of inoculated plates. Antibiotics include the following types: Streptomycin (S)10µg, Cefoxitin (Fox)30µg, Vancomycin (VA)30µg, Rifampin (RA)5µg, Penicillin G

(P)10µg, Bacitracin (B) 10µg, Ampicillin (AM)10µg, Ceftazidime (CAZ)30µg, Amikacin (AK)30µg, Erythromycin (E)10µg, Gentamycin (CN)10µg, Oxacillin (OX)1µg, Tetracycline (TE)30µg, Piperacillin (PRL)100µg, Cefoperazone (CEP)75µg, Ciprofloxacin (CIP)5µg, Imipenem (Ipm)10µg, Chloramphenicol (C)30µg, Clindamycin (DA)2µg.

Screening for the virulence factors and degrading enzymes produced by bacterial isolates: The purified and identified multi-resistant bacterial isolates were examined for their capability of producing extracellular degrading enzymes as virulence factors according to Klaenhammer, [10]. Hydrolysis of lecithin was detected on egg yolk agar (TSA supplemented with (10% egg yolk emulsion). Haemolytic activity was determined on blood agar (TSA supplemented with 10% defibrinated sheep blood). Hydrolysis of protein was detected on casein agar (TSA supplemented with 10% casein or skim milk).The plates were incubated at 37°C for 24 hours.

Extraction of DNA : The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes.

Sequencing reaction: A purified RT-PCR product was sequenced in the forward and/ or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No. 4336817.

A BLAST® analysis (Basic Local Alignment Search Tool) [11] was initially performed to establish sequence identity to GenBank accessions. The sequence reaction was done according to the instruction of the manufacture .

Phylogenetic analysis: A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, which was designed by Thompson [12] and Phylogenetic analyses were done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 [13].

Results

Distribution of collected isolates : Fifty - four bacterial cultures were isolated from infected eyes .The results in Table (1) showed that 14 (26%) have positive growth on MYP agar media and 40 (74%) have negative growth on MYP agar media .

Table (1): Specimen number and growth of bacterial isolates on MYP agar media .

No. of specimen	Growth on MYP	**No.of specimen**	Growth on MYP
1	-ve	29	-ve
2	+ve	30	-ve
3	-ve	31	-ve
4	-ve	32	-ve
5	-ve	33	-ve
6	+ve	34	-ve
7	+ve	35	-ve
8	-ve	36	-ve
9	-ve	37	-ve
10	+ve	38	+ve
11	-ve	39	-ve
12	-ve	40	-ve
13	+ve	41	-ve
14	-ve	42	+ve
15	-ve	43	-ve
16	-ve	44	-ve
17	-ve	45	-ve
18	-ve	46	-ve
19	+ve	47	-ve
20	-ve	48	+ve
21	-ve	49	+ve
22	-ve	50	-ve
23	-ve	51	+ve
24	-ve	52	+ve
25	-ve	53	+ve
26	-ve	54	+ve
27	-ve		
28	-ve		

Note: (+ve→Positive reaction, -ve→Negative reaction).

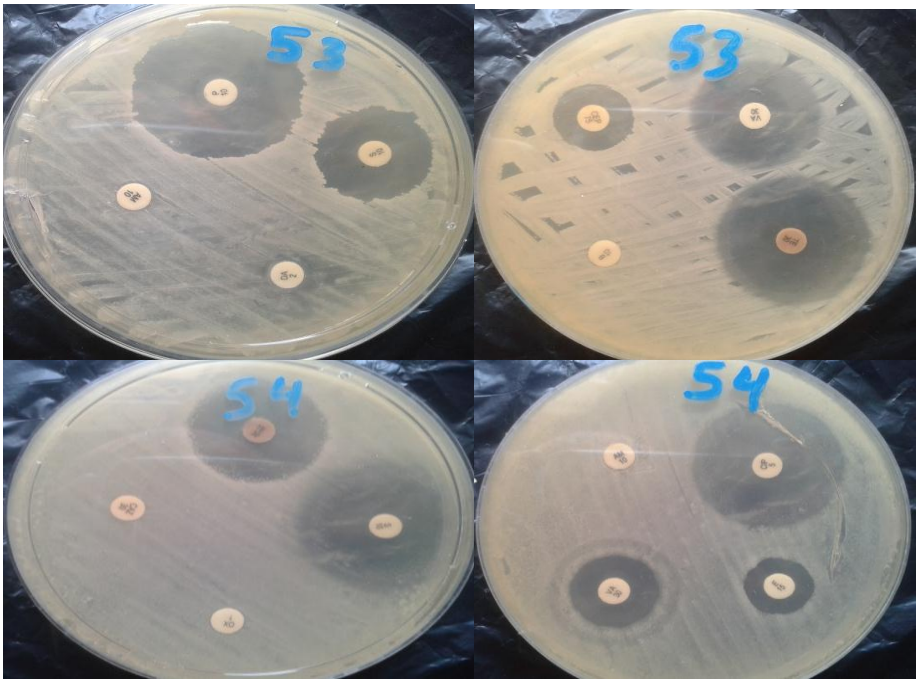
Antibiotic susceptibility test : This experiment was carried out to study the susceptibility of the isolatedbacteria towards different 19 antibiotics by using a standardized disc

diffusion method.The results in Tables (2) revealed that the tested isolates were highly susceptible to Imipenem, Ciprofloxacin and Amikacin with susceptibility percentage (100 %) , followed by Vancomycin and Penicillin G with 88% and 66 % susceptibility, respectively. On the other hand the data showed that all bacterial isolates were resistant to Cefoxifin, Ampicillin, Bacitracin, Ceftazidime, Erythromycin and Oxacillin.

Table (2): Comparative susceptibility of bacterial isolates against different antibiotics .

Antibiotic	Symbol	Conc.µg/disc	Resistant (R)		Intermediate (I)		Susceptible (S)	
			No.	%	No.	%	No.	%
Streptomycin	S	10	4	44.4	4	44.4	1	11.1
Cefoxifin	Fox	30	9	100	0	0	0	0
Vancomycin	VA	30	0	0	1	11.1	8	88.8
Rifampin	RA	5	8	88.8	0	0	1	11.1
Penicillin G	P	10	3	33.3	0	0	6	66.6
Bacitracin	B	10	9	100	0	0	0	0
Ampicillin	AM	10	9	100	0	0	0	0
Ceftazidime	CAZ	30	9	100	0	0	0	0
Amikacin	AK	30	0	0	0	0	9	100
Erythromycin	E	10	9	100	0	0	0	0
Gentamicin	CN	10	2	22.2	4	44.4	3	33.3
Oxacillin	OX	1	9	100	0	0	0	0
Tetracycline	TE	30	5	55.5	3	33.3	1	11.1
Piperacillin	PRL	100	7	77.7	2	22.2	0	0
Cefoperazone	CEP	75	7	77.7	2	22.2	0	0
Ciprofloxacin	CIP	5	0	0	0	0	9	100
Imipenem	IPM	10	0	0	0	0	9	100
Chloramphenicol	C	30	7	77.7	2	22.2	0	0
Clindamycin	DA	2	8	88.8	1	11.1	0	0

$$\%R=\frac{No.ofResistantisolates}{Totalcountofisolates}\times 100 \quad \%S=\frac{No.ofSensitiveisolates}{Totalcountofisolates}\times 100 \quad \%I=\frac{No.ofIntermediateisolates}{Totalcountofisolates}\times 100$$



Figure(1): Antibiotics susceptibility test against bacterial isolate No E53 and E54.

Screening for the virulence factors and degrading enzymes produced by selected isolates:

The selected bacterial strains were screensd for their capability of producing different degrading enzymes and virulence factors namely hemolysins, lecithinase and protease . Data in table (3) revealed that The bacterial isolates No E10,E13,E49,E53 and E54 showed positive hemolysins, lecithinase and protease production. While bacterial isolates no E2,E6,E19 and E48 were exhibited negative hemolysins and positive lecithinase and protease.

Table (3) : Screening for degrading enzymes produced by selected isolates.

Isolate No.	hemolysins	lecithinase	protease	% of Resistance
E2	- ve	+ve	+ve	52
E6	-ve	+ve	+ve	73
E10	+ve	+ve	+ve	57
E13	+ve	+ve	+ve	47
E19	-ve	+ve	+ve	52
E48	-ve	+ve	+ve	68
E49	+ve	+ve	+ve	63
E53	+ve	+ve	+ve	63
E54	+ve	+ve	+ve	73

Molecular identification of the Gram positive bacilli.

The identification of nine selected Gram positive bacilli (E2,E6,E10,E13,E19,E48,E49,E53 and E54) were confirmed by detecting of Gro-EL specific gene for Bacillus cereus. The genomic sequence of the most virulent strain Bacillus cereus E54 of the genes ; Gro-EL , hbl and Pc-plc were submitted to GenBank at NCBI web site (www.ncbi.nlm.nih.gov) in accession number MH837160 , MH844524 and MH837161 respectively.

The DNA of the selected bacteria were extracted using The QIAamp DNA Mini Kit. The groEL gene was amplified by polymerase chain reaction (PCR) using universal primers designed to amplify 533 base pair fragment of gro-EL DNA region as shown in Figure (2). The phylogenetic trees of Gro-EL, hbl and PC-plc genes were detected in Figure (3).

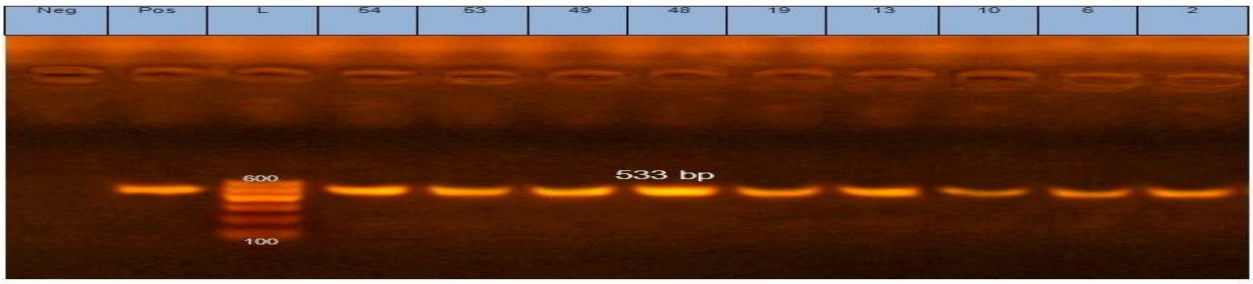


Figure (2A): Photo of agarose gel showing the absence of Gro-EL gene amplicon fragment with 533bp for all Bacillus cereus strains represented by lane2,lane6,lane10,lane13,lane19,lane48,lane49,lane53and lane54 referring to E2,E6,E10,E13,E19,E48,E49,E53 and E54,respectively including with lane (L) containing the ladder with band marker of gene fragment at 533bp as positive control standard with set of known different molecular sized fragment with more brightness intensity single band as inside lane Marker.

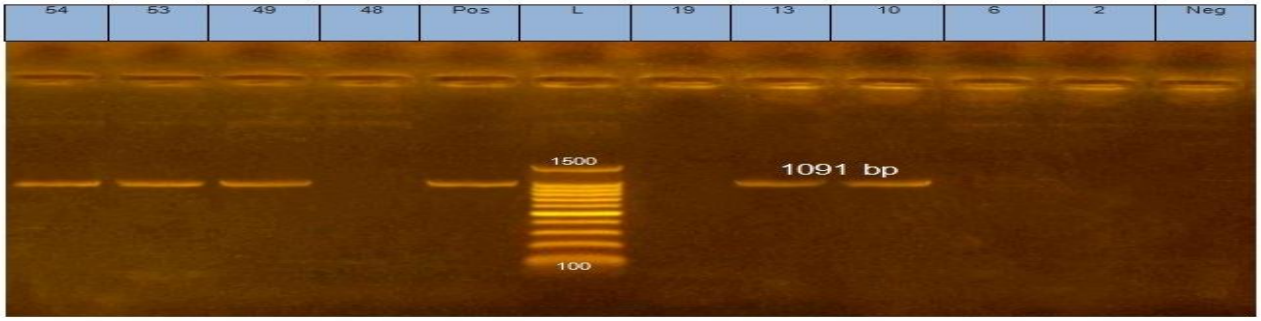


Figure (2B): Photo of agarose gel showing the absence of hbl gene amplicon fragment with 1091bp for all Bacillus cereus strains represented by lane2,lane6,lane10,lane13,lane19,lane48,lane49,lane53and lane54 referring to E2,E6,E10,E13,E19,E48,E49,E53 and E54,respectively including with lane (L) containing the ladder with band marker of gene fragment at 1091bp as positive control standard with set of known different molecular sized fragment with more brightness intensity single band as inside lane Marker.

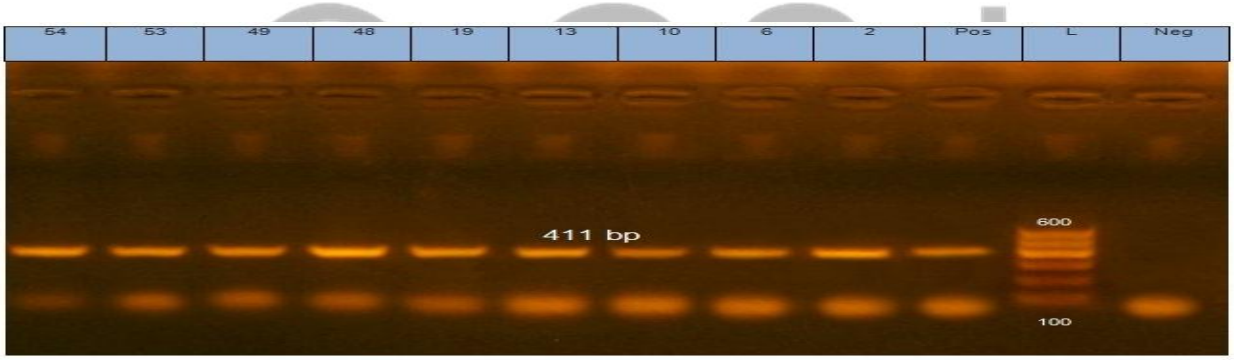


Figure (2C): Photo of agarose gel showing the absence of PC-plc gene amplicon fragment with 411bp for all Bacillus cereus strains represented by lane2,lane6,lane10,lane13,lane19,lane48,lane49,lane53and lane54 referring to E2,E6,E10,E13,E19,E48,E49,E53 and E54,respectively including with lane (L) containing the ladder with band marker of gene fragment at 411bp as positive control standard with set of known different molecular sized fragment with more brightness intensity single band as inside lane Marker.

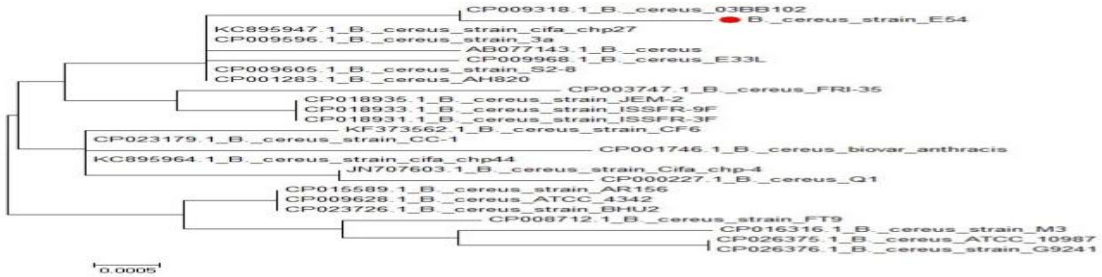


Fig.(3A):The phylogenetic tree based on partial sequencing of Gro-EL gene showing relationship neighbor-joining between B. cereus E54 (MH837161) and other closely related sequences on NCBI GenBank reference taxa.

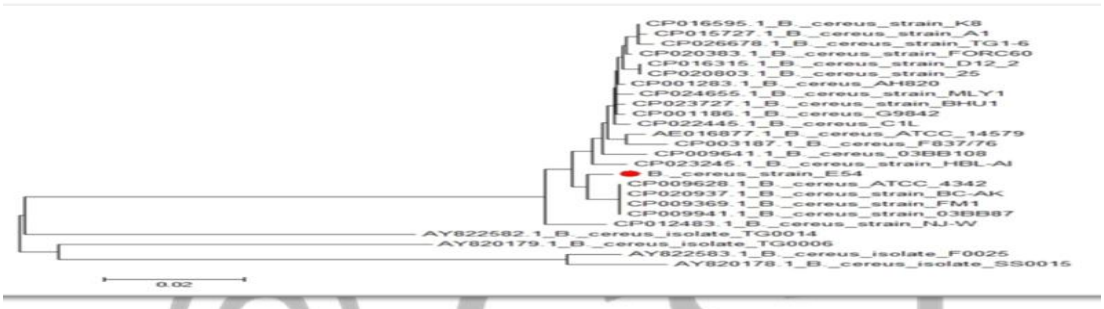


Fig. (3B): The phylogenetic tree based on partial sequencing of HBL gene showing relationship neighbor-joining between B. cereus E54 (MH844524) and other closely related sequences on NCBI GenBank reference taxa.

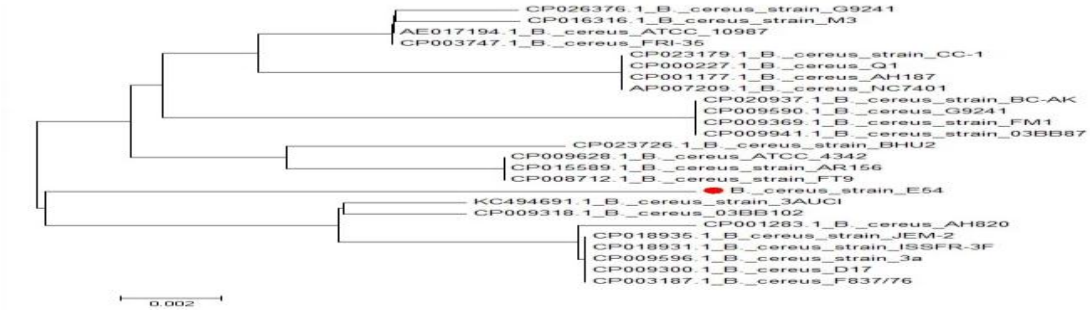


Fig. (3C): The phylogenetic tree based on partial sequencing of PC-plc gene showing relationship neighbor-joining between B. cereus E54 (MH837161) and other closely related sequences on NCBI GenBank reference taxa.

Discussion

Conjunctivitis is one of the most common non traumatic eye complaints and is one of the most frequently reported disease in the outpatient and emergency departments[14]. Infectious conjunctivitis is mainly bacterial or viral, with approximately 78% to 80% of cases being bacterial in origin [1]. This study is aimed to isolate and identify Gram positive bacilli causing eyes infection.

The present study was initiated by collection of fifty-four medical specimens from infected eyes of different patients . About 14 (26%) have positive growth on MYP agar media and 40 (74%) have negative growth on MYP agar media. These 14 bacterial isolates were selected, purified and initial morphologically identified by Gram's stain as 9 Gram positive bacilli (64%) and 5 Gram positive cocci (36). This is in accordance to the fact that, most cases of bacterial conjunctivitis are caused by Gram-positive commensal organisms (part of the normal skin flora).That is with agreement with Perkins[15] who reported results of isolates coagulase-negative staphylococci (67.8%) followed by S.aureus (23.1%). In 1995, Everett [16] reported that Gram-positive organisms accounted for 75% of the isolates. Studies conducted by researchers (Borer [17], Jeong [18], Haas [19]) have reported Coagulase-negative staphylococci as the most frequent organisms causing hospital acquired conjunctivitis, constituting 21 to 25%of bacterial isolates, Where as in the present study, the major organism isolated was *Bacillus cereus*.

In this study the antibiotic susceptibility pattern of 9 selected isolates against 19 different antibiotics was investigated by using disc diffusion method. In this study the tested isolates were highly susceptible to Imipenem, Ciprofloxacin and Amikacin with susceptibility percentage (100 %) so that it represented the most effective antibiotic followed by Vancomycin and Penicillin G with 88% and 66 % susceptibility, respectively. On the other hand, the data showed that all bacterial isolates were resistant to Cefoxifin, Ampicillin, Bacitracin, Ceftazidime, Erythromycin and Oxacillin with percentage (100%) and intermediate to Streptomycin and Gentamycin with percentage (44%) followed by Tetracycline,Piperacillin,Cefoperazone and Chloramphenicol with percentage 33% , 22% , 22% and 22% . These results are in agreement with Abo-State [20] they reported that most bacterial isolates which collected from eye drops were susceptible to

imipenem, amikacin and ciprofloxacin, while are resistance to ceftazidime, clindamycin and chloramphenicol. these strains are intermediate in its susceptibility to Gentamicin.

Dubouix [21] showed that all strains of *Bacillus cereus* which isolated from open fractures in traumatology-orthopaedy were 100% resistant to penicillin, ampicillin, amoxicillin, cefalotine,cefotaxime and imipenem, while these strain were susceptible 100% to gentamicin, petimicin,tobramycin and resistant 33% to minocycin,6% to erythromycin,3% to pristinamycin, 12% to rifampicin. Also these strain were sensitive to vancomycin and ciprofloxacin .Garcia [22]showed that Bacillus strains which isolated from topical and medicaments drugs were highly resistant to lincomycin, polyxin B and penicillin G, cephalosporin while these strains were susceptible to streptomycin erythromycin and chloramphenicol.

These antibiotics (imipenem, ciprofloxacin and amikacin) were not only effective against Gram positive bacteria but also effective against Gram negative bacteria. Itokazu [23] showed that amikacin, imipenem and ciprofloxacin were the most efficient against gram negative Bacilli which is agree with this study. Imipenem resistance rate with the Enterobacteriacea remained at the level of 1% or less. Also amikacin was broadly active against the Enterobacteriacea and Pseudomonas aeurginosa according to Lockhart [24]. The resistance of isolates to β-lactam antibiotics may be due to drug inactivation by β-lactamases like AmpC cephalosporinase (beta-lactamase enzyme that open the β-lactam ring) as an intrinsic resistance, target site modification (i.e. change in PBPs (penicillin binding proteins)) as mutational resistance and acquired resistance represented in drug inactivation [25].

The results of testing the ability of Bacillus cereus isolated from infected eyes to produce hemolysin on blood agar medium indicated that out of 9 isolates 5 isolates exhibited positive hemolysin production (55.5%), while 4 isolates (44.5%) failed to produce hemolysin (negative) . HBL is a membrane lytic system composed of the antigenically distinct proteins B, L1, and L2, encoded by *hblA*, *hblD*, and *hblC*, respectively. Strains secreting complete HBL, as demonstrated by the formation of a discontinuous zone of hemolysis around colonies on sheep blood agar plates [26].On the other side All Bacillus cereus isolated have the ability to produce lecithinase (degrading phospholipids into insoluble lipids in the form of white ppt) and protease (caseinase

degrading casein protein as extracellular degrading enzyme). Identifying the specific mechanism by which PC-PLC and HBL interact on amembrane will require significant advances towards understanding the mechanisms of both. However, our most recent model of the cause of discontinuous haemolysis provides a reasonable mechanism. The discontinuous haemolysis pattern of HBL occurs because excess concentrations of the B and L" components inhibit haemolysis [27]. The apparent mechanism is that the B and L" components self-associate at high concentrations, forming inactive homo-oligomers on the membrane surface and thus preventing the formation of competent transmembrane pores. If significant amounts of PC-PLC bind to the membrane surface without altering its character, the effect will be to reduce the membrane volume available to bound HBL components. The effective increase in component concentration on the membrane would drive the formation of inactive complexes, particularly near the diffusion source.

In addition during the present study the identification of bacterial isolates (E2,E6,E10,E13,E19,E48,E49,E53 and E54) were confirmed by using The Gro-EL gene which amplified by polymerase chain reaction (PCR) as *Bacillus cereus*. E54 Gro-EL gene sequencing is submitted to GenBank at NCBI web site (www.ncbi.nlm.nih.gov) under accession number MH837161. Hemolysin BL (hbl) and phospholipase (pc-plc) gene sequencing are submitted to GenBank at NCBI web site (www.ncbi.nlm.nih.gov) under accession number MH844524 and MH837160.

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