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Isolation and identification Streptococcus pneumonia from contact lenses of conjunctivitis and keratitis patients with bacteria resistance to antibiotics

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Abstract :

There are different methods for identification bacteria,we used culture methods,biochemical tests and molecular method,the aim of this study to decide the measure of efficient select gene for the recognition of the *S. pneumonia* by molecular tests -specific polymerase chain reaction(PCR),the assay targeting *lytA* gene from keratitis and conjunctivitis in patients and clients wearing contact lenses in several centers in Erbil city, the results showed that out of 12 investigated *S.pneumoniae* isolates by culture methods and biochemical testes ,*lytA* gene is found in 6(50%).all 6 of the positive *lytA* isolates encapsulated ,Norfloxacin and Seftazidime were the best antibiotics (100% ,83.3%) respectively, most infections was keratitis(cornal infections),in addition the majority of patients and clients were among females 78(60%),32(24.6)of patients and clients in the average age ranging from(15-20)years ,80(61.5%) of infections recoded of those who are putting cosmetic lens

المستخلص

تعد التشخيص الدقيق للبكتريا المسببة للاصابة من اهم الخطوات للوصول الى العلاج الملائم وبوقت قياسي ،توجد العديد من الطرق لتشخيص البكتريا منها التقليدية مثل التشخيص المورفولوجي والزرعية والبايوكيميائية والطرق الحديثة مثل الجزيئية،هدفت الدراسة الحالية تشخيص بكتريا *S.pneumoniae* والمعزولة من المراجعين والمصابين بالتهاب الملتحمة والقزحية بفحص جزيئي وذلك عن طريق التحري عن الجين *lytA* بطريقة تفاعل البلمرة المتسلسل PCR حيث اظهرت النتائج ان 50% اعطت نتيجة موجبة للجين وللكبسول من بين 12 عزلة لـ*S.pneumoniae* كانت مشخصة حسب الطرق التقليدية،اظهرت فحوصات الحساسية ان مضادي Norfloxacin وCeftazidime من اكفأ المضادات في الدراسة الحالية(100%،83.3%)على التوالي، وكانت نسبة المراجعين بين الاناث اعلى من الذكور(60%)78، وبمتوسط اعمار بين15-20 سنة كما كانت اغلب الاصابات هي اصابات القزحية وان (61.5%)80من العدسات المستخدمة كانت لاغراض تجميلية.

Introduction

Acontact lens is athin lens placed directly on the surface of the eye. there are various uses for it, such as therapeutic reasons for correcting vision otherwise cosmetics or for both reasons ,Many reasons leads people to wear contact lenses ,for instance some people use contact lenses to keep away from wearing glasses or to gain a more aesthetic appearance of their eyes,while others wear contact lenses for the purposes of optical reasons(Zhivov *etal*.,2007), contact lenses provide numerous advantages for example enhanced minor vision and do not collect moisture, contact lenses usually not dangerous if used appropriately, complications takes place when cntact lens used incorrectly that might going to cause for larger injuries affect the eyelid, conjunctiva cornea (John,2004), Neglecting lens care for example in appropriate wear program, lens replacement, wear it during sleeping or for too long duration, using without a doctor's prescription are a common cause of complications. which can leads to infection by avariety microorganisms including fungi or bacteria such as *Pseudomonas sp*., *Staphylococcus sp*. and *Streptococcus* that consider the most predominant pathogen ,which also are members of the normal flora of the eyelids, Bacterial keratitis in another words means a minor injury in cornea and became a devastating infection if involving bacteria that causing corneal scarring then permanently damage vision (Bharathi *etal*.,2007). *Streptococcus pneumoniae* is an important cause of keratitis (Pepose and Wilhelmus,1992) *S. pneumoniae* keratitis commonly follows surgery or trauma to the eye and is more common in patients with coexisting ocular disease Ten thousands of bacterial keratitis cases are reported in USA each year (Arnaud and Tristan,2011) ,keratitis due to *S. pneumonia* symptoms are the clear dome-shaped tissue on the front of your eye that covers the pupil and iris. This ulcerations will expand quickly, for two days. It become harder especially among immune depressed patients(Mah *etal*.,2014; Bhave and Chamie,2008) ,many signs appears among keratitis patients such urgently eye(pain, redness) excess tears,feeling that something in eye,photophobia and weak vision (Ng *etal*.,2016) Many virolence genes support *S. pneumonia* invasive for instance capsule which promoting attachment to epithelial surfaces and prevent bacteria from phagocytosis by immune system of host , *S. pneumonia* has many virolence genes such as *lytA* that encodes surface protein autolysin, represent potential targets for the specific detection of *S. pneumoniae* corresponds to a right pneumococcus (Nagai *etal*.,2001;Canvin *etal*.,1995;Berry *etal*.,1989), autolysin are endogenous enzymes that specifically degrade the covalent bonds of the cell walls(degrading peptidoglycan)(Tahereh *etal*.,2015) and eventually can induce bacterial lysis(Berry *etal*.,1989),autolysin have been postulated to play a variety of physiological roles on wall growth, wall turnover, cell separation, lysis induced by antibiotics, and pathogenicity, one of the best-characterized autolysins, the major pneumococcal lytA amidase (Feldman *etal*.,1990) the pneumococcal amidase has a modular organization, the N-terminal domain provides the catalytic function, whereas the C-terminal domain, which consists on six repeated sequences, is responsible for binding specificity to the cell wall.

There are many studies conducted the virulence of *S. pneumonia*, Sanz and his company in 1992 found out that parent

strains of *S. pneumoniae* are more virulent than mutated LytA strains, (Berry *etal*., 1989)and (Feldman etal.,1990) respectively but two main hypotheses about *S. pneumonia* virulence,the First explained that autolysis promotes the release of the intracellular toxin pneumolysin (Ply) , Ply is an important determinant of virulence and second that Ply interferes with several defense systems, including inhibition of ciliary beating.

Material and Methods

All 130 swabs samples were collected from eye infections from contact lens wearer in several eye centres in Erbil Governorate during 2016 and 2017 for ages ranging between(14-64)years old.all samples used were collected under aseptic condition and safety precautions, It were taken from patients and clients suffering from eye infections due to complications caused by wearing contact lense for both purposes (cosmetics,therapeutic),samples were taken from keratitis ,conjuctivitis and from(routine tests) healthy clients . isolation and identification of *Streptococcus pneumonia* was done according to(Versalovic *etal*.,2011) , in the clinical microbiology laboratory ,*S.pneumoniae* were detected in 9.2% (Feldman *etal*.,1990) of the samples, samples were plated onto blood base agar (5% blood red blood cells) plus chocolate agar.The plates incubated at 37°C (candle-jar), CO_2 atmosphere support hemolytic reactions furthermore provide best growth for Streptococcus. Characteristic phenotype of *S. pneumonia* (gram-positive lanceolate diplococcic.

S. pneumoniae appear as small, grey.Negative staining methods to distinguish capsular material from the bacterial cell done in agreement with (Reed *et al*.,2005) ,(Tortora *etal*.,2003) produce alpha-hemolysis (green),furthermore by using standard biochemical tests for more confirmation by catalase reaction using hydrogen peroxide , The optochin (ethylhy - drocupreine hydrochloride) susceptibility testing was performed using the standard Optochin disks are often called “P disks”(Optochin, Hardy Disk Hardy Diagnostics). Optochin sensitivity that support the diagnosis of alpha-hemolytic streptococci, P disk(5 µg) placed within the streaked area of the plate and incubate the blood agar plates overnight at 37°C (in a candle-jar),if the inhibition zone near the P disk is 14 mm or greater indicates sensitivity,all isolates were examined by Bile solubility test(sodium deoxycholate), bil spot test HDx, Deoxych 10%, 15 ml, Hardy Diagnostics,US.using direct plate method that by putting a drop of bile Spot Reagent near a suspected colony(18-24 hour old) softly turn round the drop over several representative colonies and incubated for 30 minutes. appearance of a hemolytic zone in the medium at the sight where the colony was located is bile soluble and indicates a positive test (Versalovic*etal*.,2011).

Antimicrobial susceptibility testing was performed using the standard Kirby-Bauer diskdiffusion method on Mueller-Hinton agar (LAB ,England) ,Antibiotic discs (Becton Dickinson)and(Bioanalyse,France) guidelines from the Clinical Laboratory Standards Institute (CLSI, 2013). The following antimicrobial agents were tested : Amikacin (AN) , Gentamycin (GM), Augmentin(AC), Carbenicillin (Car), Piperacillin (PIP), Cefotaxime(Cef), Ceftazidime (CFX) , Azterionam(AT), Norfloxacin (NOR). *E. coli* ATCC25922 was used as acontrol .

Preparation of DNA templat

Atotal 130 strains of Streptococcus isolates were cultured on LB broth,for over night phenol-chloroform technique utilized for DNA extraction and the determination the purity and concentration as before described by (Pospiech and Neuman,1995)

Amplification of *lytA* gene were performed in 25 µL volume reaction mixtures

Detection of virulence genes by PCR.PCR amplification mixtures and lasB oprI. oprL. toxA. exoS.
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Detection of virulence genes by PCR.PCR amplification mixtures and lasB oprI. oprL. toxA. exoS.
Detection of virulence genes by PCR.PCR amplification mixtures and lasB oprI, oprL, toxA, exoS,

(corporation promega,UAS) this contain(0.3 µl 1.5 U Taq DNA polymerase, 1.5 µl of dATP, dCTP, dTTP and dGTP and 5 µl PCR buffer,) , (1µM) forward and revers primers(table 1) and 3µl of template DNA (bacterial cell suspension), deionized Sterile distilled water was added to make a final volume of 25 µl.

followed by PCR product was electrophoretically separated on agarose gel (1%, containing 0.5 µl/ml ethidium bromide.

PCR programe amplifications were carried out in thermocycler using the program as previously described by (Tahereh *etal*.,2015), The oligonucleotide sequences of primer used in this study are scheduled in Table 1.

Table (1): Nucleotide Sequence of Primer Chosen to detect *lytA* Gene For PCR

Oligonucleotide	Sequence	Expect product Size (bp)
***LytA* primer forward**	F: 5'-CAA CCG TAC AGAATG AAG CGG-3'	319bp(Sourave *etal*.,2010)
***lytA* primer reverse**	R: 5'-TTA TTC GTG CAA TAC TCG TGC G-3'	

Result

Out of 130 samples obtained from keratitis and conjunctivitis ,three types of pathogens were isolated, The overall result shows Pseudomonas species has the highest isolation rate 14(10.8%), followed by *S.pneumoniae* 12(9.2%) and Staph species has 3(2.3%) as showen in Table(2), this study focused on streptococcus pathogen, 12(9.2%) of isolates were identified *S. pneumoniae*

through a characteristic spherical or ovoid form and appeared achain of two(diplococci) or more bacteria cocci which grow mostly in pairs and in chains of cells, α-hemolytic colonies, Catalase negative, Optochin susceptible, Bile soluble. Confirmation was also provided by PCR targeting the *S. pneumoniae* specific *lytA* gene (*S. pneumoniae* species specific), out of 12 investigated *S. pneumoniae* isolates, *lytA* gene were found in 6(50%) as shown in figures (1,2) , The incidence of *S.pneumoniae* respect to the gender and age groups of the patients and clients was found to be more in females Table(3), in the age group (male and female) between (ten – twenty) years with contact lens for cosmetic purposes as shown in figure (3) and Table(3). The percentage of *S. pneumoniae* in order with site of infection; keratitis 5(42%) > both infection(keratitis,conjunctivitis)3 (25%) > (16.5%)for each of periodic checks and conjuctivitis as given in Table (2,3).6(50%) of isolates encapsulate distributed as follows,2(33.3%),for each of the cnjuctivitis , keratitia and periodic checks while none encapsulate were detected in (conjunctivitis and keratitis) isolates as given in Figure(2) .

The *S. pneumoniae* isolates were more susceptible to Norfloxacin (100%), Ceftazidime (83.3%), Azterionam (75%), Amikacin ,Augmentin and Cefotaxime (66.7%) ,Also *S. pneumoniae* isolates were less susceptible to Gentamycin (41.7%),while isolates appeared resistant to each of Carbenicillin and Piperacillin as shown in Table (4).

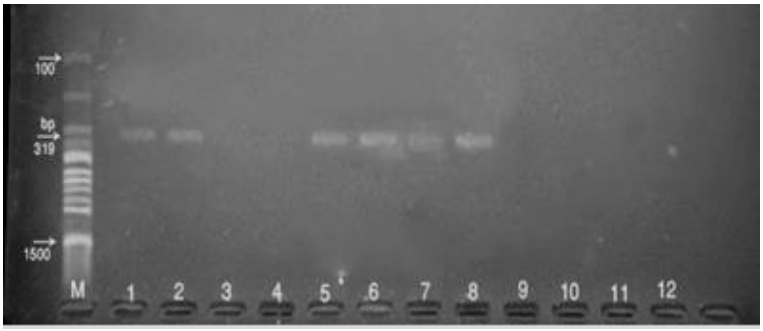

Fig.(1): agrose gel electrophoresis with positive PCR amplification of 319 bp fragment of lytA gene from DNA of *S. pnumonia* isolates from different samples .,1) 100 bp marker., 2, 3,5,6,7,8) *S. pnumonia* (positive results for *lytA*)., (3,4, 9, 10, 11,12) *S. pnumonia* without *lytA* gene.

Table(2):Types of isolates from infection sources

Sites of infection		No.ofsamples	Positive *S.pneumonia*				Infected with other micro organisms		
			No.	%	Capsule			No.	%
					No.	%			
Keratitis		82	5	42	2	33.3	Staph.,Pseudomonas	2,6	47.2
Conjuctivitis		10	2	16.5	2	33.3	Staph.,Pseudomonas	1,3	23.5
Both infections	**Keratitis**	10	3	25	-	-	Pseudomonas sp.	4	23.5
	Conjuctivitis								
Other infections		3	-	-	-	-	-	-	-
Periodic checks(healthy clients)		25	2	16.5	2	33.3	Pseudomonas	1	5.9
Total		**130**	**12**	**100**	**6**	**100**	**-**	**17**	**100**

Table (3): Isolation rate of *S.pneumoniae* from different samples of patients and clients.

The purposes of using contact lens	No. of tested samples	Female			Male		
		Age	No.	%	Age	No.	%
Therapeutics	50(38.5%)	40-64	35	27	50-62	15	11.5
Cosmetics	80(61.5%)	14-65	43	33	13-40	37	28.5
Total	**130**	**-**	**78**	**60**	**-**	**52**	**40**

Table (4): explanation of antimicrobial sensitivity testing for all *S. pneumonia* isolates

Antimicrobial disk	Antibiotic sensitivity of *S. pneumonia*					
	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Amikacin(AN)	2	16.7	2	16.7	8	66.7
Gentamycin(GM)	3	25	4	33.3	5	41.7
Augmentin(AC)	-	-	4	33.3	8	66.7
Carbenicillin(Car)	10	83.3	2	16.7	-	-
Piperacillin(PIP)	10	83.3	2	16.7	-	-
Cefotaxime(Cef)	-	-	4	33.3	8	66.7
Ceftazidime(CFX)	-	-	2	16.7	10	83.3
Azterionam(AT)	2	16.7	3	25	9	75
Norfloxacin(NOR)	-	-	-	-	12	100

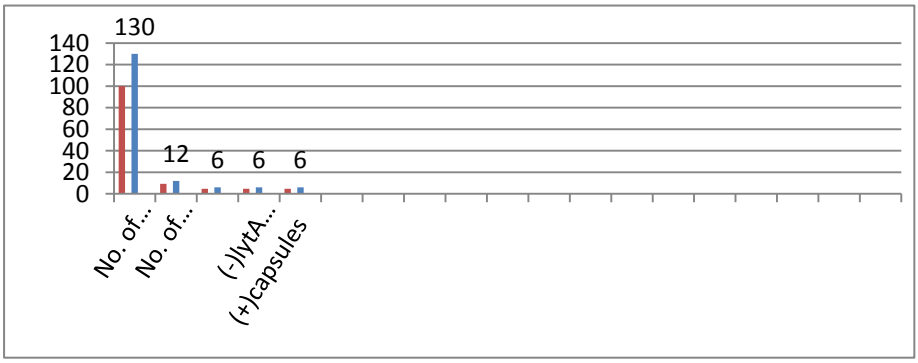

Fig.(2): number and percentages of the positive *S.pneumonia* ,*lytA* gene and capsule samples.

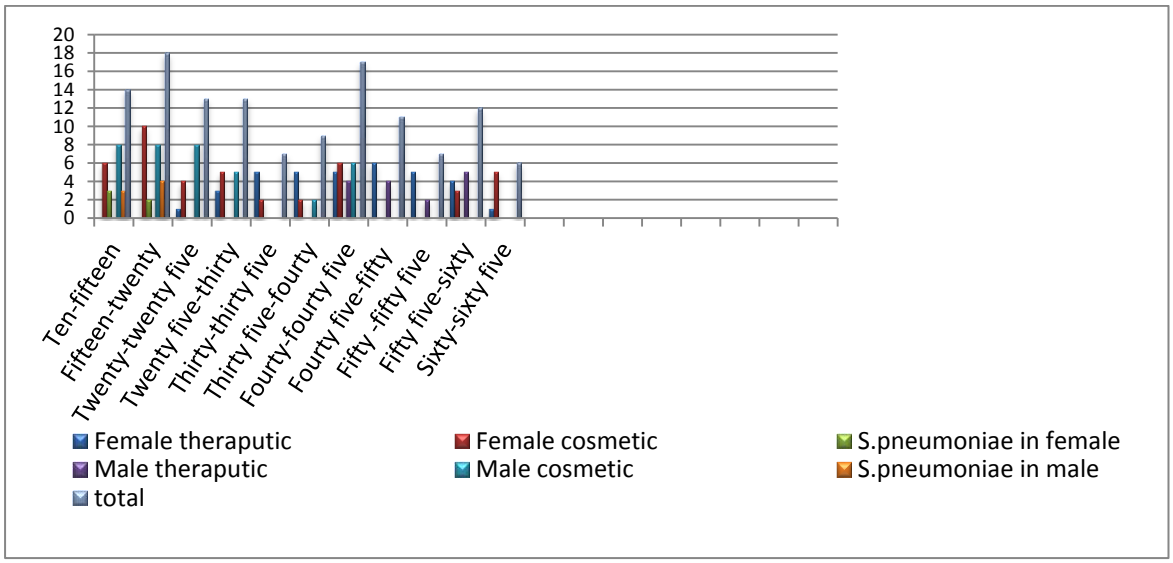

Fig.(3):Ages and genderof patients and clients with the purposes of contact lens using

Discussion:

S.pneumoniae is a member of human microbial flora, set up on the mouth, pharynx, it causes a variety of diseases including keratitis and conjuctivitis, *S.pneumoniae* is considered the top causes of bacterial keratitis (Ng *etal*.,2016 ; Bhave and Chamie, 2008). *S.pneumoniae* usually don't cause any hurt. But the combination of a lot of them on our contact lenses and any small scrape on our eyes can be very dangerous. *S.pneumoniae* easily spread to eyes by hands.

Identification the responsible microorganism that cause conjunctivitis and keratitis an important steps for the ophthalmologist and the treatment will be in a correct way, phenotypic characterization such as metabolic enzymes ,optochin test as well as bile solubility of the *S.pneumoniae* strains were present in some pneumoniae-mitis, pseudopneumoniae strains, traditional tests doesn't give a precise diagnosis .

Our study classified *S.pneumoniae* in second rank with little difference (two isolates) further than *Pseudomonas* that not in agreement with(Green *etal*.,2008) which have concluded *Pseudomonas* are the most microbes were isolate in eye infections among lenses wearers followed by *Staphylococcus. Streptococcus* in third world countries is the most common pathogen isolated from keratitis , While largely infection types in developed countries are lachrymal sac or of conjunctival blistering and Pseudomonas as well as staphylococcus is the mainly microbes isolated.

This study showed that an encapsulated isolates were exist in half cases50%, which is the most important virulence factors in *S. pneumoniae*,in same time 50% of cases caused by noncapsolated *S. pneumoniae* which means in addition to the capsule it is other pathogenic factors are required by *S. pneumoniae* for virulence (Arnaud and Tristan,2011;Kelly *etal*.,1994) , some

previous studies have different conclusions, In a study conducted by(Erin *etal.,* 2002;Reed *etal*.,2005;Norcross *etal*.,2010) they have showen the polysaccharide capsule does not seem important to play arole in the infection ,so it is non-essential for keratitis,but they disagreed with present study from side keratitis isolates was encapsulated while cojucative non encapsulate,though they showed that an encapsulated strain was capable of establishing conjunctivitis in a rabbit injection model, 50% of current study gave positive result to *lytA* gene(319bp) which stimulate inflammation (Brayn *etal*.,1992;Blue *etal*.,2003), whereas (Thomas *etal*.,2012) showed 100% of their isolates positive result(295-bp).they showed also that *lytA* gene is specific to *S. pneumoniae* with the exception of bile-insoluble pneumococci.,also the method published by Sheppard *et al*. (2004) targeting the *lytA* gene constitutes a sensitive and specific assay for distinguishing *S. pneumoniae* from its close relatives in the mitis group. This is due to differences in the *lytA* gene sequence of *S. pneumoniae* and the other mitis group streptococci. This study suggests that the bile-insoluble pneumococcal strains test negative in the *lytA* gene PCR and (Daniel *etal*.,2006)study proved that *S.pneumoniae* harbored typical *lytA* alleles (927-bp-long) they result that detection *lytA* gene by polymerase chain reaction (PCR) assay permits fast and reliable recognition of accurate *S. pneumoniae* strains as well as characterizes an improved diagnostic tool for the study of pneumococcal.it also save time and effort greater than the classical culture method especially when the sample is blood, cerebrospinal fluid, or pleural fluid (Berry *etal*.,1989;Versalovic *etal*.,2011;Thomas *etal*.,2012;Tahereh *etal*.,2015).

virulence factors are different among *S.pneumoniae* strains that cuses different diseases,due to differences in the genetic material that took place in many ways for example *S.pneumoniae* strains that eye infections has gentetic profile different from that adapted for lung infections or tonsils of the same host,may be the reasons returned to different in adaption period and changes occur in that period such transformation between normal flora and *S.pneumoniae* which leads to exchange genetic information with other bacteria,another reasons that *S.pneumoniae* genom is containing *BoxB* elements which spreded in multiple copies which gives varying gene expression as well as plasticity (Aguiar *etal*.,2008) ,(Mogens *etal*.,2008).

Pham and his group in 2006 recommend Ceftazidime and Vancomycin as the initial treatment and then alternated every hour without interruption even at night for the first day, vancomicine, levofloxacin, penicillin, and cefotaxime were best antibiotics in their study,in another study conducted by Parmar and his group in 2006 recomended quinolones anthbiotics for keratitis infections mainly that goes back to cocci,the above studies compatible with present that resulted Norfloxacin and Ceftazidime were best antibiotics.

Infectious keratitis and conjuctivitis affects both males and females. Afemale preponderance in this study(60%) that was agreed with (Keay *etal*.,2006;parmar *etal*.,2007).

In addition(61.5%) of patients were wearing cosmetics lens has been noted,may be due overload attention by women in external appearance,most infection recorded in the teenager group, that concluded age has important role to influence the aetiological agent, may that returns to ignorance hygiene matters and neglect by wearers such as lack of affirmation to visiting the eye doctor at least once a year, sleeping or napping with contacts in, and swimming while wearing contacts. not replacing lenses as often as prescribed, not regularly replacing storage cases and less likely to be instructed on appropriate lenses use and basic hygiene rules(Richard *etal*.,1991;Arnaud and Tristan,2011).

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