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Antibiotic Susceptibility of Different Isolates from Ophthalmic Patients

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ABSTRACT

***Introduction:** External and intraocular infections can lead to visual impairment which is a major public health issue. Bacteria are the most frequent pathogens affecting ocular structures. On the other hand the increasing rate of antimicrobial drug resistance is a worldwide concern. The aim of current study was to estimate the occurrence of bacterial ocular infections, their antimicrobial susceptibility patterns and the resistance of bacteria by PCR in ocular infection.*

***Methodology:** A hospital based cross-sectional study was conducted from January-December 2017 at Al-Shifa Trust Eye Hospital, Rawalpindi. Ocular specimens from blepharitis, conjunctivitis, keratitis, endophthalmitis, periorbital cellulitis and dacryocystitis were collected from 50 individuals with suspected ocular infections. Data on socio-demographic and risk factors was also recorded using a Consent form. Data analysis was performed using SPSS (version 21.0) and confidence interval 0.05 with was considered statistically significant.*

***Results:** Among 50 study subjects, 7 subjects infected with Staphylococcus aureus showed resistance to oxacillin. Among samples infected with Streptococcus pneumoniae, 10 showed resistance to Moxifloxacin. The subject infected with Pseudomonas aeruginosa, 9 patients showed resistance to Ceftriaxone. PCR showed the presence of ESBL gene in the resistant genes.*

INTRODUCTION

Bacteria are unicellular microscopic organisms commonly found all over the environment. They comprise positive and negative effect on environment and human health. They cause variety of local and systemic infections in human and animals. (Woese *et al.* 2012). It can be classify by number of ways; however the major criteria are Gram Staining. Under this basis, they separated in to gram +ive and gram -ive bacteria. Classification based on gram staining, usually structural variation in cell wall depends upon the amount of peptidoglycan they have. In spite of their peptidoglycan, gram +ive bacteria are more prone to antibiotics than gram-negative, due to absence of external membrane (Madigan *et al*. 1997).

Bacteria cause infections that are threat for human and animal health. These infections may be local and systemic. The infections of eye like (conjunctivitis, Blepharitis, Cellulitis, Endophthalmitis and Keratitis) infection of ear, leprosy, etc. (Voigt *et al*. 2015). In the current project, we will lay focus on micro-organisms that cause ophthalmic infections. There are some bacteria like (Gram +ive bacteria) *Staphylococcus aureus, staphylococcus epidermidis, Corynebacterium, Streptococcus;* (Gram -ive bacteria) *Pseudomonas* and *Moraxella* species that are concerned in ophthalmic infections during bacterial attack in a variety of ways (Scott *et al*. 2016). Conjunctivitis is inflammation of conjunctiva includes infection from various bacteria and viruses. It is also known as "Pink Eye" a

frequent, extremely communicable eye infection. Only thirty percent of basic care patients with conjunctiva infections are recognized to have bacterial conjunctivitis, even though eighty percent are cured with antibiotics (Rietveld *et al.* 2007). Blepharitis is ordinary condition that causes redness of eyelids. The situation may be tough to control as it leans to persist. Tears play an important role to maintain cornea healthy; tear film dilemma may make people extra at threat for infections of cornea (Gadaria-Rathod *et al.* 2013). Cellulitis is swelling of eyelid skin. An infection of eyelid skin is called Preseptal Cellulitis that does not expand outside the orbit. Orbital Cellulitis is inflammation of the orbit. Orbital cellulitis is usually more severe than Preseptal cellulitis (Wasserman *et al.* 2007). Endophthalmitis, the infection penetration in interior of eye with bacterial Endophthalmitis, sightlessness could result without immediate cure, frequently by use of valuable antibiotics, about 4 to 8% risk of Endophthalmitis (Moshfeghi *et al*. 2007). Keratitis is the infection of cornea, frequently this infection commonly found in people using contact lens. Keratitis is most severe barrier when using contact lens. In dreadful cases, it may go to scarring of cornea that damages the vision (Gopinathan *et al*. 2009).

According to McMenamin P.G, the ocular immune systems protect eye from infection and control the therapeutic processes against injuries. The interior of eye lacks lymph vessels although it is extremely vascularized and several cells of immune system exist in uvea containing mostly macrophages, mast cells and dendritic cells. Cells of Immune system contest against infections of eye. Immunologically, eye cornea is very particular tissue which continuous contact to outside world means that it is susceptible to broad variety of microorganisms, whereas its wet mucosal surface creates cornea mostly prone to violence. Furthermore, McMenamin PG stated that absence of vasculature and comparative immune disconnection from rest of entire body creates immune protection tough. Cornea is the eye's refractive control to serve as an obstacle to stay pathogens away from the rest of eye. Immune responses in the cornea approach from close vascularized tissues and innate immune responsive cells that located in the cornea. For ocular defenses both innate and acquired reactions are necessary (McMenamin 2012).

Antimicrobial resistance is ability of a microbe to resist the effects of drug. Mechanism of resistance in bacteria are; (i) bacteria yield enzymes that destroys the antibacterial agent before it touches its target, (ii) alteration in drug target; (iii) cell wall becomes impermeable (Li and Nikaido 2009). Infection due to resistant microbes is progressively more difficult to treat, requiring higher doses which may be more costly or more toxic. Antimicrobial resistance causes millions of deaths every year (Shimizu *et al*. 2013). The steady rise in rates of resistance of many vital pathogens, containing MRSA (Methicillin-resistant *Staphylococcus aureus*), VRE (Vancomycin-resistant *Enterococcus*), MDR (Multidrug-resistant) *Pseudomonas aeruginosa*, poses a risk to public health (Meyer *et al.* 2010). ESBL (Extended-spectrum β-lactamase) manufacturing pathogens and Methicillin-resistant *Staphylococcus aureus* are prevailing in several hospitals throughout the world (Cantas *et al*. 2013).

According to Shanmuganathan, the amount of MRSA in optical *Staphylococcus aureus* infections from one institution differ from 3 percent to 30 percent, some outlines showing greatest cause of MRSA (Shanmuganathan et al. 2012). According to Asbell PA, the Surveillance Network, which screens Antimicrobial Susceptibility outlines of bacteria in the U. S., stated an rise in the amount of MRSA *Staphylococcus aureus* optical infections, from 29.5 percent in 2000 to 41.6 percent in 2005, which disclosed MRSA is a increasing threat in optical field (Asbell et al. 2008). Currently, ESBL producing pathogens and MRSA infections are also progressively more noticed in population and local hospitals managed by physicians who first treated the patient (Otter and French 2010)

MATERIALS AND METHODS

Samples (n=50) were collected from Department of Microbiology, AL-Shifa Trust Eye Hospital, Rawalpindi by pre-sterilized swab during January 2017 - June 2017. The study design was approved by the Ethics Committee for Research on Human Subjects, University of Veterinary and Animal Sciences, Lahore, Pakistan. Each Sample was marked with specific Lab number, patient age, gender etc…. samples were processed to identify the infected pathogen. Each sample was inoculated separately at Blood Agar and Nutrient Agar plate. Inoculated plates were aerobically incubated at 37°C for twenty four hours. After incubation, bacterial colonies were identified on the basis of colony morphology (shape, color,

translucency, surface elevation and margins), gram staining and biochemical characteristics.

Quantity of Antibiotic per discs

Name of Antibiotics	Quantity of Antibiotic per discs
Vancomycin	30ug
Levofloxacin	5ug
Tobramycin	10ug
Oxacillin	1ug
Moxifloxacin	5ug
Ofloxacin	5ug
Ceftazidime	30ug
Ciprofloxacin	5ug
Ceftriaxone	30ug
Amikacin	30ug
Cefuroxime	30ug

Patterns of antibiotic resistance of isolated bacterial pathogens will be studied. By using disc diffusion method, antibiotic resistance patterns will observed (Bauer *et al.* 2008). The tests of antimicrobial susceptibility of all isolates were performed as per guide lines given by Clinical and Laboratory Standards Institute (CLSI 2012). Multiple antibiotics (at least one from each group) were tested to detect the antibiotic resistance in isolates. The antibiotics used for Sensitivity Testing were Vancomycin, Levofloxacin, Tobramycin, Oxacillin, Moxifloxacin, Ofloxacin, Ceftazidime, Ceftriaxone, Ciprofloxacin and Amikacin.

An isolated bacterial colony of *Staphylococcus*, *Streptococcus* and *Pseudomonas* from the Blood agar and Nutrient Agar plate was plucked with a sterilized loop and 0.5 McFarland suspensions was prepared in PBS (Mcfarland and L'engle 2008). Then spreading of inoculums was done in four dimensions by rotating the plate at 90° after swabbing half plate with the help of sterilized swab stick.

Then with the help of multi-disc dispenser, antibiotic discs were applied on the Muller-Hinton agar plate. The plates were aerobically incubated at 37°C in incubator for twenty four hours. After incubation, the diameter of the inhibition zones of these antibiotics was calculated in millimetres with the help of metric ruler, according to the "Clinical and Laboratory Standards Institute" guidelines (Ericsson and Sherris 2017).

DNA Extraction

To isolate the DNA, bacteria was cultured in 10 ml Lauria broth (LB) at 37°C for 24 hours. Pellet was form at bottom at 6500 resolutions/minute at 4°C for five minutes. Then pellet was wash with 2000 µl TEN buffers and centrifuged at 6500 resolutions/minute at 4°C for five minutes. The supernatant was removing and the pellet was break down. In pellet 1000 µl of SET buffer and 100 µl of lysozyme was add. Mix it until the mixture was become homogeneous. The samples were place in incubator at 37°C for 30 min. This was kept on by the supplement of 500 µl of TEN buffers and 50 µl of 25% SDS (Flip-flop the 15 ml falcon tube until lysis was occurred) with incubation at 60°C for 10 min in water bath. 100µl of 5 M NaCl was mix after the mixture was cool. The DNA was handled with equivalent ratio of phenol and chloroform (1:1). Mixed the suspension and centrifuged at 6500 resolutions/ minute for twenty five minutes at 4°C. Supernatant was separate and shifted into new 15 ml falcon tube. Equal volume of chloroform was added and centrifuged at 6500 resolutions/ minute for twenty five minutes at 4°C. Again supernatant was separate and transfer into new 15 ml falcon tube. The DNA was precipitate by the treating double volume of chilled absolute ethanol. Centrifugation was done at 6500 resolutions/minute for ten minutes. Finally washed the walls of the 15 ml falcon tube by 70% ethanol and centrifuged at 6500 rpm for 5 min. After drying the 15 ml falcon tube 50 µl of 1X TAE buffer was added in the tubes and properly mixed. The samples were name properly and stored at -20°C (Sambrook et al. 2014).

Quantitation of DNA

Quantitation of DNA was done by Nanodrop method (Thermo Scientific). 1ul of DNA was placed on cuvette and reading was obtained by running instrument. For good PCR product 30-50ng of DNA must be in 1ul of sample. In case of higher DNA quantity, it was diluted by addition of autoclaved distilled water.

Agarose Gel Electrophoresis for DNA

Total DNA was observed in 0.9 % Agarose gel electrophoresis (BIO-RAD). About 100 ml of 0.9 % By dissolving 0.9g of Agarose in 1X TAE buffer (prepared by mixing 10 ml of 50X TAE buffer prepared Agarose gel in 90 ml of distilled water made up the volume up to 100 ml. Heated the Agarose in oven for 1 minute to melt completely.

Than cooled up to 50ºC. Then 13µl of Ethidium bromide (50 mg/ml) was added in it and mixed. Agarose gel was dispensed into the properly sealed caster and a comb of required well size was inserted. Put the gel for 15 to 20 minutes to solidify. The comb was removed after

solidification. The casting tray was positioned in electrophoresis tank which contains 500ml of 1X TAE buffer. 6µl of loading dye (6X) was added to 5µl of DNA. Run the gel at 120 volts for 30 minutes. Bands of total DNA were detected by using Ultra Violet light.

PCR for the Amplification and detection of CTX-M gene

For PCR, 12 samples were used and PCR was done under optimized conditions using programmable Thermal cycler (BIO-RAD). A reaction mixture of 25ul was prepared by using following components of following concentrations. The components that were used for PCR reaction, Master Mix 12.5ul, forward primer 0.6ul, Reverse Primer 0.6ul, Template DNA 2ul and Sterilized water 9.3ul.

Concentration of PCR reagents

Solution	Component
Master Mix	12.5ul
Forward primer	0.6ul
Reverse primer	0.6ul
Template DNA	2ul
Sterilized water	9.3ul

Conditions for PCR

Initial denaturation	94°C for 2 minutes
Denaturation	94°C for 45 sec
Annealing	52°C for 45 sec
Elongation	72°C for 45 sec
Final elongation	72°C for 10 minutes

The reaction was started with an initial denaturation temperature at 94°C for two minutes, which was followed by 30 cycles with denaturation temperature of 94°C for forty five seconds, annealing temperature of 52°C for 45 seconds and elongation was completed at 72°C for 45 seconds. At the end, final elongation was done at 72°C for 10 min. CTX-M gene was amplify and detected by using the CTX-M gene specific designed primer by PCR (Kiratisin et al. 2008).

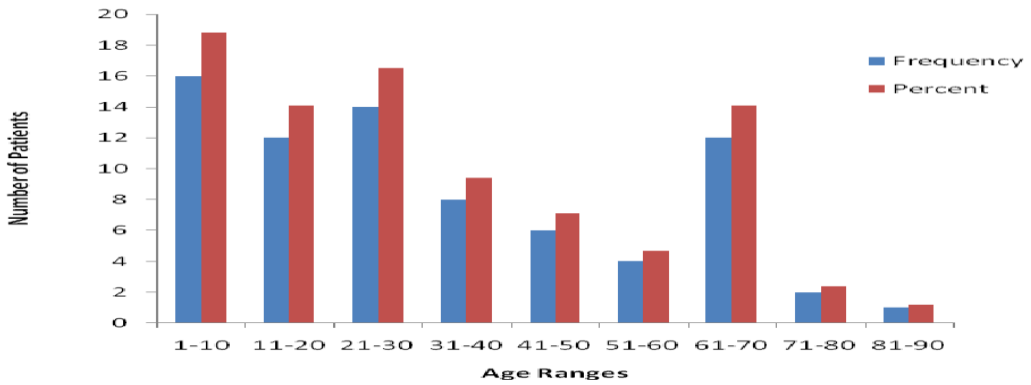
Beta lactamase CTX-M primers

Target	Primer Name	Sequence	Reference
Beta-lactamase CTX-M	CTX-F	TCTTCCAGAATA AGGAATCCC	(Kiratisin et al. 2008)s
	CTX-R	CCGTTTCCGCTA TTACAAAC	

Agarose Gel Electrophoresis of PCR Product

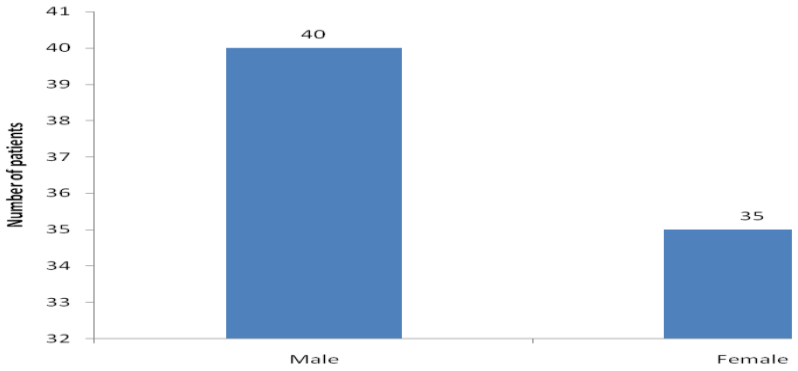
Amplification of gene was observed by Agarose gel electrophoresis (BIO-RAD). For this 5 µl of 6X loading dye was added to 25 µl PCR products. 6 ul of 1 kb plus DNA ladder was added. Hundred milliliters (100 ml) of 2 % Agarose gel was prepared in 1X TAE buffer (prepared from 50X TAE). Amplified products were loaded along with 5 ul of 1 kb plus DNA ladder in a well and the gel was run for 40 minutes at 80 volts. UV Transilluminator was used to visualize the amplified product. Data on socio-demographic and risk factors was also recorded using a Consent form. Data analysis was performed using SPSS (version 21.0) and confidence interval 0.05 with was considered statistically significant.

RESULTS



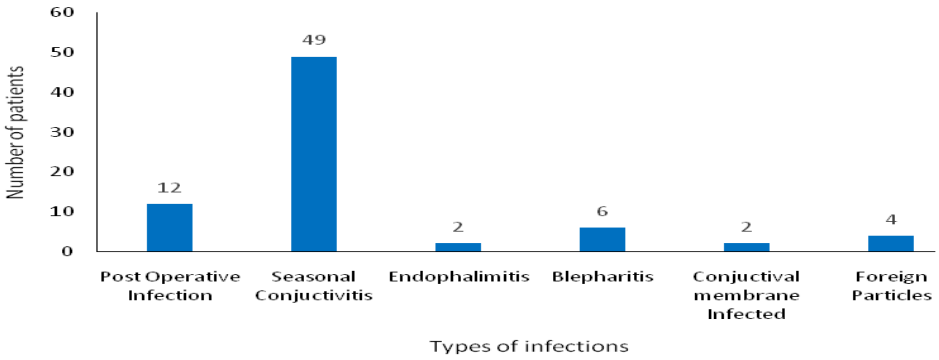
Graph showing number of patients in each age group

The total number of patients was divided in to 9 groups on the basis of their age. e.g. the age of patient included in group 1 was 1-10 years(16), in group 2 the age of patient range between 11-20 years (12),there were 14 patients were included in age range of 21-30, the age range 31-40 of patients included were 8 , there were 6 patients of age ranging 41-50, age range of 51-60 patients were 4 , 12 patients were present in age range of 61-70, 2 patients were included in age group of 71-80 and the age range of 81-90 having 1 patient were included.

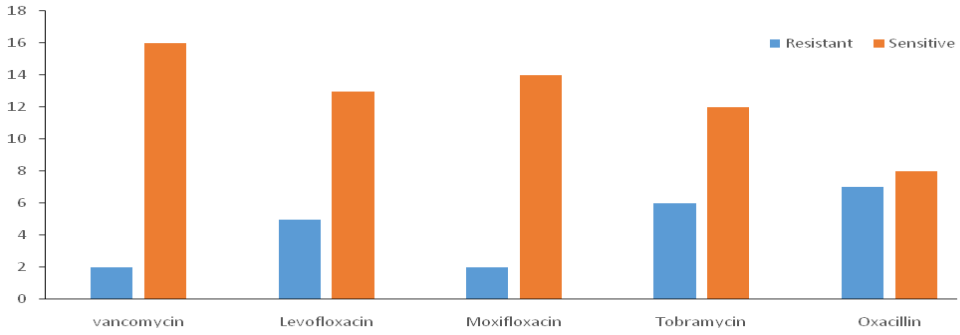


Graph shows distribution of patients with respect to gender

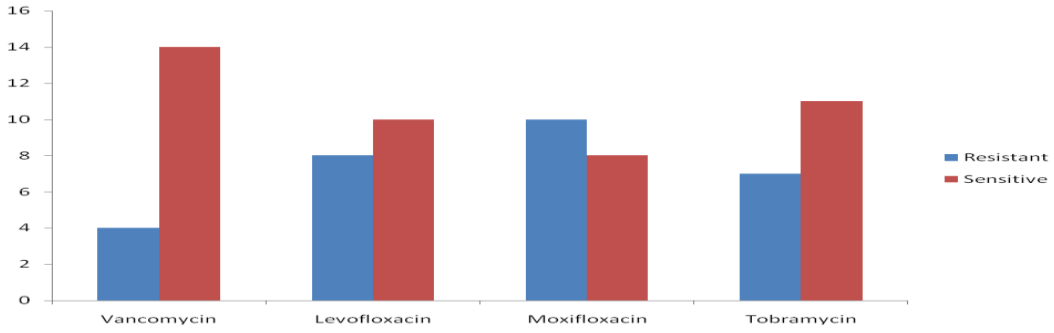
There were 40 males and 35females in the study. Gender ratio was 1.14:1. Gender ratio was non-significantly different between the two sexes ($\chi 2$= 0.333; p < 0.564). There were 12 patients (14.1%) having post operative infections, 49 patients (57.6%) were seasonal conjunctivitis, 2 patients (2.4%) whose have endophamiltis, 6 patients (7.1%) whose blepharitis, 2 patients(2.4%) are included as conjunctival membrane Infected and the 4 patients (4.7%) were infected by foreign particles.



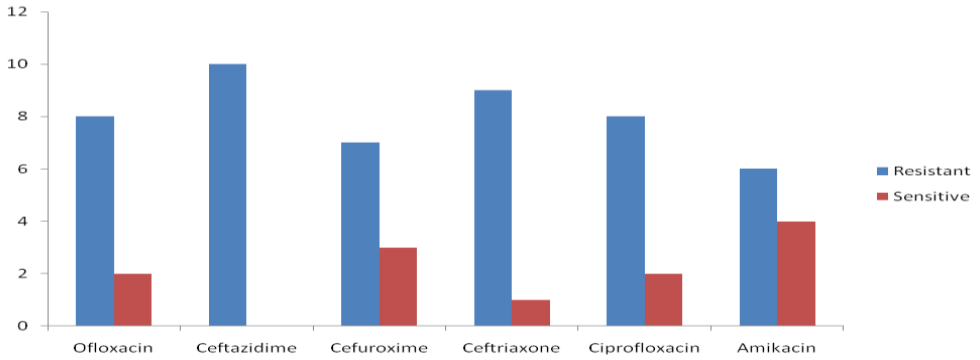
Graph distribution of patients with respect to type of Infection



Graph shows the distribution of Antimicrobial activity of Staphylococcus aureus



Graph shows the distribution of Antimicrobial activity of Streptococcus pneumoniae



Graph shows the distribution of Antimicrobial activity of Pseudomonas aeruginosa

Total DNA was isolated from isolates of *Streptococcus pneumonia, Pseudomonas aeruginosa* and *Staphylococcus aureus* and agarose gel was run to see the extracted DNA. Nanodrop method was also used to check the amount of DNA. Fig 4.53 shows the bands of the extracted DNA from ESBL producing *Streptococcus pneumonia, Pseudomonas aeruginosa* and *Staphylococcus aureus.*

By using programmable thermal cycler, amplification of *CTX-M* gene was done by applying different conditions. The reaction was started with an initial denaturation temperature at 94°C for two minutes, which was followed by 36 cycles with denaturation temperature of 94°C for forty five seconds, annealing temperature of 52°C for 45 seconds and Elongation was completed at 72 °C for 45 seconds. At the end, final elongation was done at 72°C for 10 min.0.9 % Agarose gel electrophoresis was run of PCR products. Bands were seen at 909 bp.

Amplification of CTX-M gene by PCR

DISCUSSION

Bacteria are most common agents that cause eye infections in human(Derek S 2008). Conjunctivitis is the most ordinary ocular infection in Europe, North America and Egypt (Weiss et al. 2008). Earlier studies showed that most common ocular infections are conjunctivitis (46.3%) and least common are dacryocystitis (25.7%) Shahani et al. (2012) reported that conjunctivitis is most widespread eye infection in Sharkeya Governsate; it is highly prevalent among pre-school children (63%). In the current study, seasonal conjunctivitis was most prevailing type of ocular infection (57.6%).

The prevalence of conjunctivitis was found more among male patients where as higher cases of endophamiltis (2.4%) observed among female patients in the current study. Previous studies have recommended that patients with eyelid abnormalities such as blepharitis (29.4%) and ectropion are at higher risk for increasing post- operative endophthalmitis (Scott et al. 2012). The increase in the number of eyelid and conjunctival bacterial flora in these patients as well as increase number of multi resistant flora that these patients anchorage (de Kaspar et al. 2012). In the present study, the post operative cases were (14.1%) and blepharitis (7.1%).

In earlier study, infection proportion of Gram positive showed 68.1%. Contrasting strains of *S. pneumoniae* that can cause pneumonia, bacteremia, or meningitis, *S. pneumoniae* lacks a capsule and seems to be well modified to the ocular environment (Martin et al. 2011). Sherwal and Verma (2008) showed that Gram positive cocci are mainly common isolates among notorious and opportunistic pathogens.

The infection proportion of Gram positive bacteria were the dominate isolate (76%) in the current study. This was also supported by other studies conducted in India and Nepal suggestive of Gram positive cocci was most important cause of external ocular infection (Upadhyay et al. 2009). Among the Gram positive bacteria, CoNS (Coagulase negative *Staphylococci*) was the most predominant pathogen with an overall prevalence in the present study (32%). From the previous reports, *S. pneumoniae* strains caused outbreaks of bacterial conjunctivitis in Unites States. From the previous reports, Methicillin Resistant Coagulase Negative *Staphylococci* (CoNS) have been reported the most commonly isolated pathogens from patients with acute bacterial conjunctivitis (Leibowitz 2010).

In the current study, most of Gram negative isolates were sensitive to Amikacin but resistant to ceftriaxone and ciprofloxacin, this was very similar to previous report from Gondar (Epling J et al. 2012). Several reports also showed similar patterns of drug resistance among Gram negative bacteria. *Pseudomonas* species was among the highly drug resistant isolates reported previously. *P. aeruginosa* has now obviously emerged as a leading nosocomial pathogen, because of its ubiquitous nature, ability to survive in moist environments and resistance to enormous bulk of antimicrobial drugs. Many other reports proposed that the CTX-M-type ESBLs can now really be the greatest ESBL type throughout the world (Khater and Sherif 2014). The OXA-type β-lactamase is called due to their oxacillin-hydrolyzing capabilities. They mostly happen in *Pseudomonas aeruginosa* but have been noticed in several other G. -ive bacteria. The OXA-type ESBLs were initially found in *Pseudomonas aeruginosa* isolates in Turkey. The development of ESBL OXA-type β-lactamase from parent enzymes with finer ranges has several parallels with the progress of SHV- and TEM-type ESBLs (Weldhagen 2009).

However, the occurrence of Extended-spectrum beta-lactamase (ESBL) resistant *P. aeruginosa* has been growing over recent years. In the present study, the ESBL production was changeable from 57.6% patients who got infection during summer season to 14.1% patients having post-operative infections showing a high difference in the frequency of ESBLs production. When an ESBL is suspected in *P. aeruginosa*, PCR based molecular techniques may help to identify the gene. Another previous study, all ESBL producing isolates were subjected to PCR to detect ESBL genes, including *bla* $_{SHV}$, *bla* $_{CTX-M}$, *bla* $_{TEM}$, *bla* $_{GES}$, *bla* $_{PER}$ and *bla* $_{VEB}$ gene. DNA sequence analysis of PCR products revealed that the strains carrying *bla* $_{SHV}$ were identical to *bla* $_{SHV-12}$ and *bla* $_{TEM}$ were identical to *bla* $_{TEM-24}$. Among *P.aeruginosa* producing ESBLs, 30 strains (14.3%) had *bla* $_{CTX-M-1}$ gene, 27 strains (12.9%) contained *bla* $_{CTX-M-3}$ gene, 29 strains (13.8%) carried *bla* $_{PER}$ gene, 24 (11.4%) strains had *bla* $_{VEB}$ gene and 20 strains (9.6%) contained *bla* $_{CTX-M-2}$ gene, respectively. In the present study, 6 (12%) strains were positive for Ctx-m gene.

In current study, total DNA was isolated from isolates of *Streptococcus pneumonia, Pseudomonas aeruginosa* and *Staphylococcus aureus* and agarose gel was run to see the extracted DNA. Nanodrop method was also used to check the amount of DNA. Amplification of CTX-M gene of *pseudomonas aeruginosa* was done by applying different conditions of thermal cycler and size of bands were seen at 909 bp in 1% Agarose gel electrophoresis tray.

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