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Microbiological Profile of Ocular Infections: In Reference to their Resistance and Biofilm Formation

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Abstract: The eye and its associated structures are distinctive predisposed to infection by the various microorganisms. External & internal infections can lead to visual impairments, which is a major public health problem. Bacteria are the most frequent pathogens affecting ocular structures; the increasing rate of antimicrobial drug resistance is a worldwide concern. The prevalence and severity of infection depends on the site of infection and type of infectious agents. The present study was aimed to determine the incidences of bacterial and fungal ocular infections and to assess the antibiotic susceptibility pattern of these etiological agents. Clinical samples were collected from various hospitals/clinics of Surat since December 2018. From total collected clinical samples 87% of samples produced positivity. Many of them were polymicrobial. From positive cases, associated pathogens were isolated and identified based on standard microbiology procedures and predominance of Gram Positive Cocci 38 (51.35%) were observed, followed gram negative short rods 26 (35.13%) and 10 (13.51%) cases of fungal infections. Isolates were further tested for their antibiotic susceptibility patterns against commonly used antimicrobials. Association between type of infections and etiological agents was also studied. Bacterial & Fungal isolates were predominantly isolated from all the ocular cases includes conjunctivitis, corneal ulcer, keratitis, blepharitis, dacryocystitis, scleritis and endophthalmitis but higher cases of fungal infections were observed in corneal ulcer. Conjunctivitis was the dominant ophthalmic disease followed by corneal ulcer. Early access to clinical and microbiological diagnosis with appropriate treatment can prevent the ocular morbidity and mortality.

Keywords: Ocular Infections, Bacterial & Fungal isolates, Antibiotic Susceptibility pattern, Multidrug resistance.

I. INTRODUCTION

Eye is the most important sensory organ concerned with the perception of vision. It is a unique organ that is impermeable to almost all external organisms and is also aided with a number of defense mechanisms, if these barriers are broken, infection may occur. From birth and throughout the human life, a very small number of bacterial commensals are found on the conjunctiva of the eye. The eye may be infected from an external source or through intraocular invasion of microorganism by blood stream. While the anterior segment is infected by direct invasion from the anterior route, blood-borne infections may reach the posterior segment of the eye. Even what may be considered a minor infection elsewhere in the body can be "fatal" to the eye in terms of visual compromise [1], [2].

Infections can be mono or poly-microbial and is also associated with many factors including contact lenses, trauma, surgery, age, dry eye state, chronic nasolacrimal duct obstruction and previous ocular infections [3], [4].

Conjunctivitis is the inflammation of the bulbar (covering the globe of the eye) and tarsal (lining the orbit) conjunctiva caused by bacterial infections, trauma, or autoimmune disease. Patients will complain of redness, and a foreign body sensation that is often associated with discharge. Most cases of bacterial conjunctivitis resolve spontaneously in a week to 10 days [5], [6]. Keratitis is inflammation of the cornea with or without violation of its epithelium constitutes to keratitis. Patients will present with an acutely red, painful eye and often complain of foreign body sensation, tearing, and vision change. Contact lens is the main cause of keratitis. Corneal abrasions may accompany a keratitis because of excessive rubbing or scratching of the affected eye. Prompt diagnosis, treatment, and identification of cause are paramount to prevent vision loss due to ulceration, necrosis, and scarring [7], [8]. Infectious endophthalmitis is defined as an inflammation of intraocular tissues or fluids secondary to intraocular infection. Colonization of organisms inside the eye can occur through introduction of infectious agents into the eye following a breach in ocular barriers or by dissemination through the systemic blood stream. Endophthalmitis are categorized according to underlying

cause as: (a) Postsurgical: acute, and delayed or chronic; (b) post traumatic; (c) bleb-related; and (d) endogenous: fungal, bacterial, and other. When infectious agents reach vitreous cavity across an opening in the globe, it is termed as exogenous endophthalmitis and when it occurs by hematogenous spread, it is termed as endogenous endophthalmitis. Exogenous endophthalmitis usually occurs following surgical or traumatic alteration of structural integrity of the globe. Occasionally, exogenous endophthalmitis results from contagious spread of infectious microbes from ocular adnexa, especially following infections on the cornea or sclera [9], [10], [11]. Scleritis a severe destructive disease, sometimes leading to the loss of an eye from deteriorating vision, severe pain, or even (occasionally) perforation of globe. Such changes, when occur, are rapid and therefore an early diagnosis and effective treatments are essential. Onset of scleritis is usually gradual, building up over several days. Blepharitis is a condition that is caused by bacterial infection on the surface of the eyelids and lashes. It can cause chronic infection and inflammation to the lids and surface of the eye. Treatments generally start with eyewashes to clean the skin surface of bacteria and other debris, and may include prescription drops or ointments. Symptoms include red, itchy eyelids that may look greasy and crusted. Dacryocystitis is an inflammation of the lacrimal sac, which usually occurs because of obstruction of the nasolacrimal duct [12].

Ocular infections, if left untreated, can damage the structures of the eye leading to visual impairments and blindness. Even though the eye is hard and protected by the continuous flow of tear which contains antibacterial compounds, inflammation and scarring once occurred may not be easily resolved and requires immediate management [13]. The etiology and antibiotic resistant patterns may vary with geographical location according to the local population [14]

Most predominant bacterial pathogens causing ophthalmic infections include *Staphylococcus aureus*, *Coagulase negative staphylococci*, *Genus Streptococcus*, *Corynebacterium*, *Bacillus*, *Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Nocardia*, *Non-fermentors* and in others group of bacteria [15], [16], [17].

Despite the protection by the components of tear in protecting the ocular surface from various pathogens along with the blinking action of the eyelids, the resident bacteria of the conjunctival sac or the environmental bacteria establish infection resulting in the need of antibiotics intervention to treat the disease [18].

Although treatment guidelines for these ocular infections recommend that laboratory culture and smear tests be conducted, when possible, for determination of the causative pathogens, in practice the initial choice of antibiotic therapy is generally made without knowing the identity or susceptibility of the ocular pathogen [19], [20].

The major route of infection is by the entry of conjunctival bacterial flora at the time of surgical procedure. The most widely applied method to reduce the rate of ocular infection following surgery is by the application of topical povidone-iodine or the antimicrobial agents preoperatively to reduce microbial flora around the eye [21], [22]. The broad-spectrum antibiotic therapy for the bacterial infections is initially used in order to prevent a decline in the vision or permanent vision loss that may require surgical intervention [23]. Blindness has an impact not only on the life of the affected person but also on the individual's family and the society as a whole. As per the WHO estimates, 39 million out of 285 million visually impaired people worldwide are blind, with preventable causes being as high as 80%. India being a developing nation has a large share in the scenario with 8 million of 62 million visually impaired people being blind [24]. The optimal choice of preoperative topical antibiotics depends on many factors, including the isolated bacteria, their antibiotic susceptibility and resistance patterns, rapidity of action, rate of penetration, and toxicity [25]. Repeated exposure of ocular flora (microbes living on or inside the body), however, may select for resistant bacterial strains and cultivate 'superbugs' with multiple-drug resistance that may considerably affect the treatment of ocular infections caused by such bacteria into the eye [22]. Frequent or inappropriate, systemic long-term use of an antibiotic may also result in the development of bacterial antibiotic resistance [20]. With this background this study was undertaken to detect the bacterial and fungal profile of the different forms of ocular infections and also assess the antimicrobial susceptibility pattern of bacterial and fungal isolates at our institute in order to come up with concrete information for physicians and policy makers who deal with ocular infections to know the microbial profile and antibiotic susceptibility.

II. MATERIALS AND METHODS

A. Study design

We have studied Non – repetitive 85 various clinical samples collected from patients suffering from eye infections and on consultation in various Private and Government Hospitals of Surat, Gujarat from December 2018 to March 2019.

B. Sample collection

Ocular samples were collected from Conjunctivitis, Corneal Ulcer, Dacryocystitis, Blepharitis, Keratitis and Endophthalmitis diagnoses. An ophthalmic surgeon collected specimens from all patients with the sterile swabs.

C. Specimen transportation

The respective specimens after collection immediately transferred into Brain-Heart Infusion (BHI) Broth. Tubes were tightly capped, gently mixed, labelled within 3 hours (WHO, 2009) were transported to the Microbiology Laboratory at Shree Ramkrishna Institute of Computer Education and Applied Sciences, Surat for microbiological analysis.

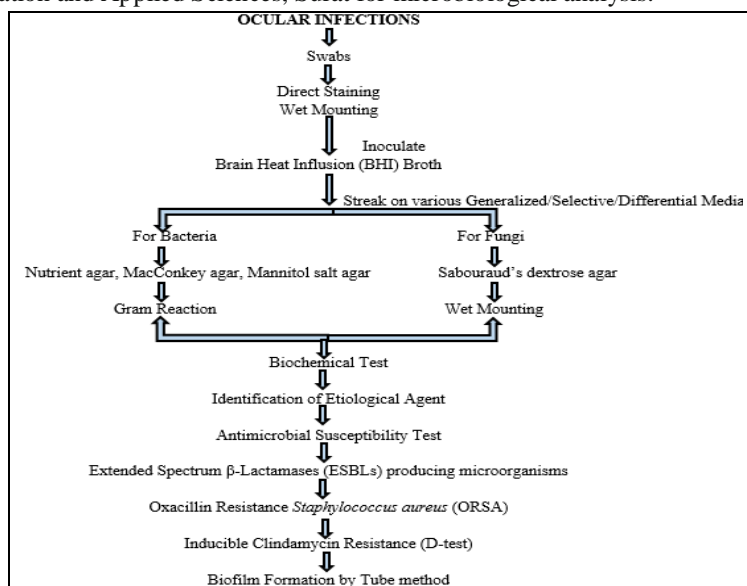


Fig.1 Working flowchart of Microbiological processing

D. Microbiological processing

- 1) *Isolation of Isolates:* Immediately after sample collection, Direct Gram staining (for bacteria) and Wet mount (for fungi) was performed from all samples. The samples were then inoculated on to Brain heart infusion (BHI) broth and transported to the microbiology laboratory of Ramakrishna. A loopful of sample was streaked aseptically on Nutrient agar plate, MacConkey agar plate, Mannitol salt agar plate (MSA) for selective isolation and differentiation of bacterial pathogens and for fungi on Sabouraud's dextrose agar plate (SDA). After incubation period (24/48 hours for bacteria and 4-5 days for fungi), samples observed as positive in case of growth.
- 2) *Identification of Isolates:* All isolates were confirmed and identified from their morphological, colonial, Growth and biochemical characterization using standard references (John G. Holt, Bergey's manual of Determinative bacteriology, 11th edition, and Jean F. Macfaddin, Biochemical tests for identification of medical bacteria, 3rd edition).
- 3) *Biochemical profiling of Isolates:* The biochemical profiling was carried out by performing various biochemical tests viz. Indole production test, Methyl Red test (MR)-Voges Proskauer test (VP) test, Citrate utilization test, H₂S production test, Urea hydrolysis test, Gelatin liquefaction test, Catalase test, Coagulase test, Oxidase test and TSI agar slant. All the media were inoculated with the loop full of culture by aseptic transfer technique or stabbing technique. The inoculated test media were incubated at 37°C for 18-20 hours.
- 4) *Antimicrobial Susceptibility Test:* Antibiotic sensitivity was done for bacterial & fungal isolates using Kirby-Bauer disk diffusion method using discs of standard potency. The results were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines [CLSI document M44-A (CLSI 2008)].

E. Detection of Extended Spectrum β-Lactamases (ESBLs) producing microorganisms

The worldwide prevalence of extended spectrum beta lactamase producing *Enterobacteriaceae* is increasing making the need for optimization detection technique. The screening of ESBL producer was done according to criteria recommended by the CLSI guidelines. For each test contacting cephalosporin alone (Ceftriaxone, Cefoperazone) and in combination with Sulbactam are applied. Zone of inhibition were measured following overnight incubation aerobically at 37°C. The inhibition zone around the cephalosporin disc combined with Sulbactam is compared with the zone around the disc with Cefoperazone alone. The test is positive if the inhibition zone diameter is ≥ 5mm larger with Sulbactam than without.

F. Detection of Oxacillin Resistance *Staphylococcus aureus* (ORSA)

Inducible resistance to oxacillin was tested as per CLSI guidelines (2009). 0.5 McFarland turbid inoculum of well isolated colony of *Staphylococcus* spp. From the plates incubated previously was prepared and inoculated in M.H. agar plates. After prediffusion time of 15 minutes the oxacillin disc (1µg) were placed on medium with sterile forceps. Plates were incubated at 37°C for 24 hours. After incubation measure the diameter of zone, which ≤ 10 mm considered as resistant.

G. Detection of Inducible Clindamycin Resistance (D-test)

This test is necessary because some bacteria express a phenotype known as MLSB, in which susceptibility tests will indicate the bacteria are susceptible to clindamycin, but *in vitro* the pathogen displays inducible resistance. Inducible resistance to clindamycin was tested by 'D-test' as per CLSI guidelines. 0.5 McFarland turbidity inoculum of well isolated colony of *Staphylococcus aureus* from the plate incubated previously was prepared and inoculated the Muller Hinton Agar plates. After pre diffusion time of 15 minutes the clindamycin (CLI) disc (2µg) and Erythromycin (ER) disc (15µg) discs were placed 15mm apart edge to edge manually with sterile forceps. Plates were incubated at 37°C for overnight and the plates were observed for the flattening of zone (D-shaped) around clindamycin in the area between the two discs that indicated inducible clindamycin resistance.

H. Detection of biofilm formation by Tube method

1) *Biofilm formation by Bacterial isolates*: Tube method is a qualitative method for biofilm detection. A loopfull of test organism were inoculated in 10ml of Trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 hours. After incubation tubes were decant and wash with phosphate buffer saline (pH 7.3) and drained properly. Tubes were then stained with 0.1% safranin solution. Excess stain was washed with distilled water. Tubes were then dried in inverted position. The visual scoring for tube method was done. Biofilm formation was considered strongly positive when a visible thick film lined the wall and bottom of the tube. If thin film were lined then it is considered as a weakly positive and there were no line formation consider as negative result. Tubes were examined and the amount of biofilm formation was scored as none/Weak, Moderate and High.

2) *Biofilm Formation by Fungal Isolates*: A loopful of test organisms was inoculated in 10ml of Sabouraud's dextrose broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 hours. After incubation tubes were decant and wash with phosphate buffer saline (pH 7.3) and drained properly. Tubes were then stained with 0.1% safranin solution. Excess stain was washed with distilled water. Tubes were then dried in inverted position. The scoring for tube method was done according to the results of the control. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. Tubes were examined and the amount of biofilm formation was scored as none/Weak, Moderate and High.

3) *Microscopic Observation of Biofilm*: Microscopic observation of biofilm was performed. The smear was prepared from the biofilm specimen from tube method. Showing biofilm formation. As per standard protocol of gram staining was observed under oil immersion objective.

I. 16S r RNA Sequencing of Screened Isolate

Molecular identification and classification on the basis of 16S r RNA sequence analysis is important for correct identification of microbial species. Therefore, the highest antimicrobial resistance and biofilm formation was selected and identified as *Staphylococcus capitis*. The results of sequence was submitted to National Centre for Biotechnology Information (NCBI) Gene Bank.

III. RESULTS & DISCUSSION

A total non-repetitive 85 samples of eye infection samples was collected from various hospitals of Surat, Gujarat from time duration of December 2018 to March 2019.

All isolates were reconfirmed again from their morphological, colonial and biochemical characteristics using standard references (John G. Holt, Bergey's manual of Determinative bacteriology, 11th edition, and Jean F. Macfaddin, Biochemical Tests for Identification of Medical Bacteria, 3rd Edition).

A. Positivity of Samples

In present study as per sample size calculation 85 of eye infection swabs were collected. Among the 85 collected samples, 74 cases showed positivity and suspected as cases of infections.

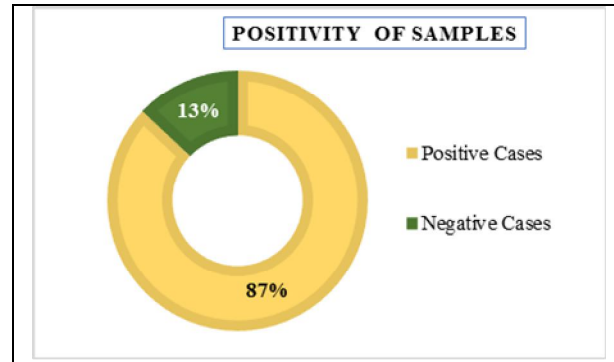


Fig.2 Percentage of culture positivity in ocular cases.

As indicated in the above (fig.5.1) 87% eye show positive cases and 13% show negative cases. In contrast to our study 51% positivity and 49% negative cases was reported by S. Rajesh et al., (2017).

B. Distribution of Samples as Per Age and Gender Wise

We collected 85 clinical samples and distributes them as per the age and gender of the patients.

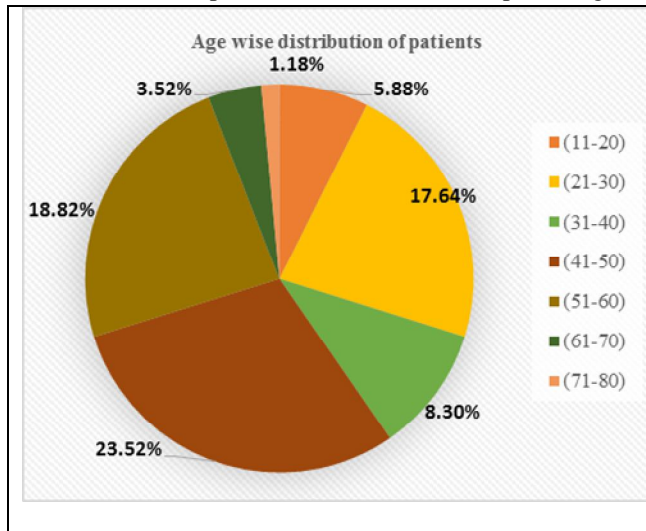


Fig.3 Age wise distribution

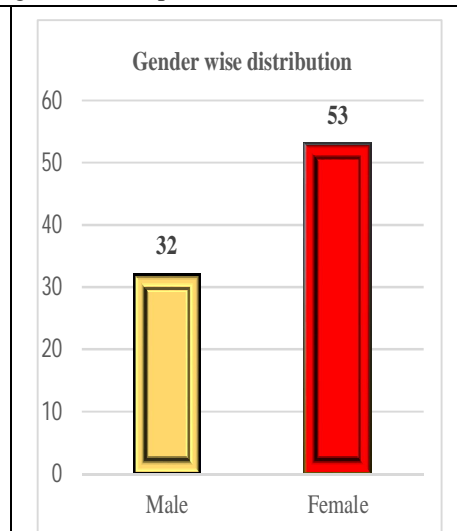


Fig.4 Gender wise distribution

C. Distribution of Samples as Per Eye Infections

Further, collected samples were distributed as per frequency of type of eye infection.

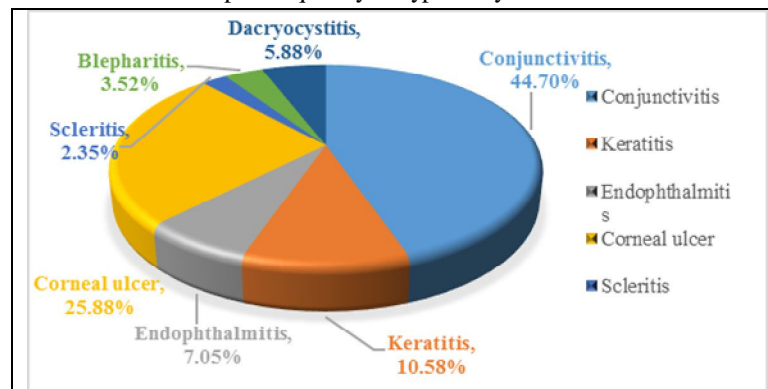


Fig.5 Percentage of Eye infection studied.

In contrast to our study Leela Rani K. et al., (2018) reported conjunctivitis as the predominant infections (accounting up to 63%) of the total cases. In present study conjunctivitis was (44.70%) found as prevalent infections amongst all followed by corneal ulcers.

D. Distribution of Isolates as per Etiological Agent

From the result of morphological, colonial and growth characterization, isolates were successfully identified. The most predominant Gram negative etiological agent we found in bacterial cases was *Staphylococcus aureus* followed by Gram negative *Escherchia coli* and in fungal cases *Aspergillus niger*.

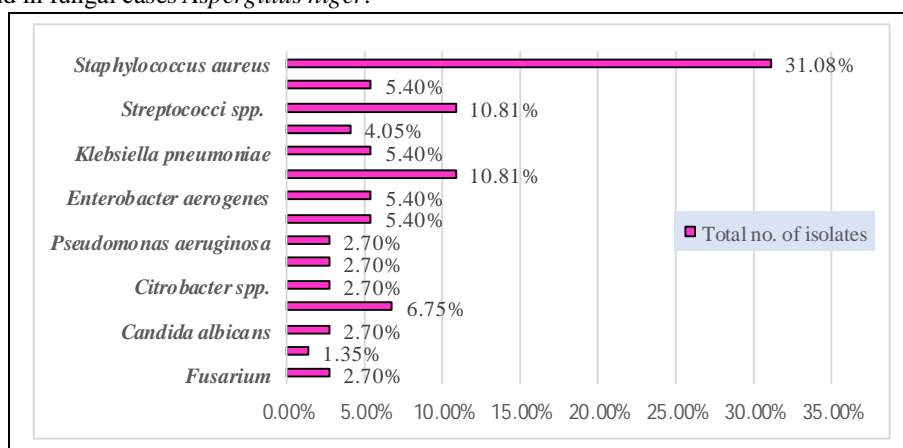


Fig.6 Distribution of different etiological agent as per frequencies.

E. Antibacterial Susceptibility Profiles of Isolates

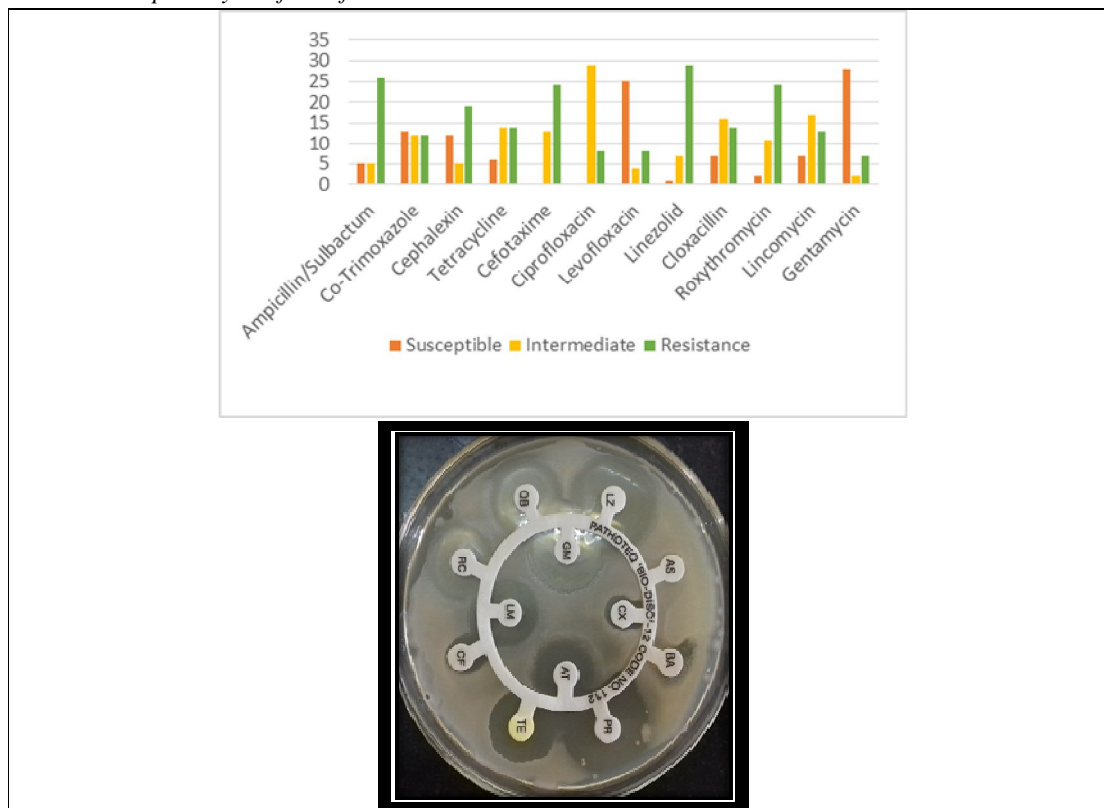


Fig.7 Resistogram of Gram Positive isolates

According to Mebrahtu Teweldemedhin et al., (2017) Gentamicin (89.1%) showed the highest resistance pattern. In the present study, higher resistances observed against the Gram positive group of isolates resist to Linezolid (57.89%) and Gentamycin (47.36%) compare to other antibiotic.

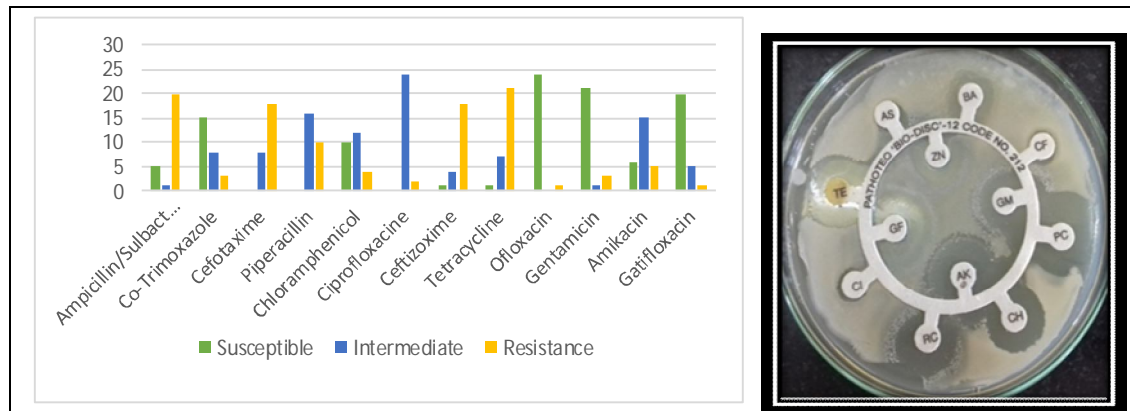


Fig.8 Resistogram of Gram Negative isolates

According to Mebrahtu Teweldemedhin et al., (2017) Ofloxacin (87%) showed the highest resistance pattern. In the present study, higher resistances observed against the Gram negative group of isolates resist to Ofloxacin (80.76%) compare to other antibiotic.

F. Antifungal Activity of Isolates

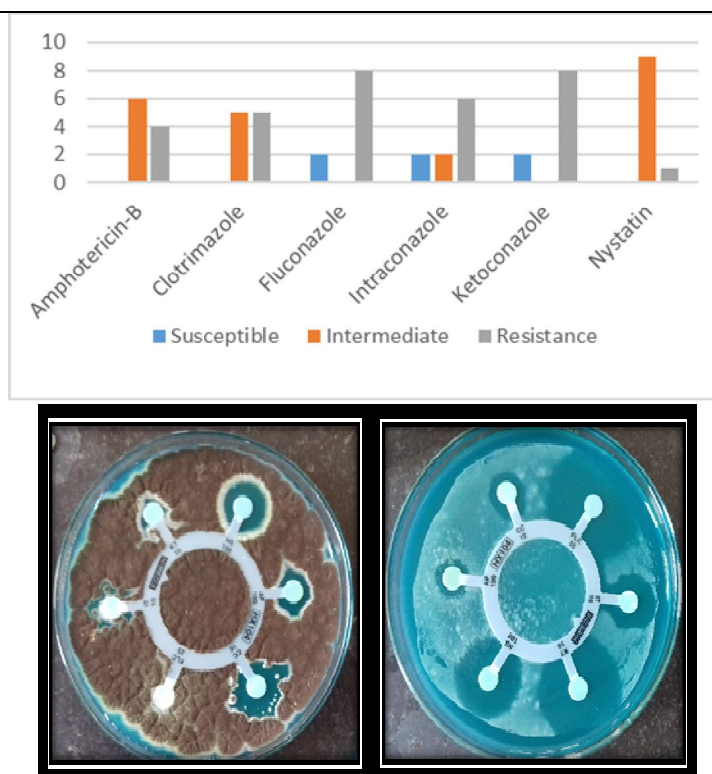


Fig.9 Resistogram of fungal isolates

In the present study Antifungal Activity by Clotrimazole, Fluconazole and Ketoconazole showed the highest resistance among the fungal isolates.

G. Detection of Multidrug Resistance (MDR)

Multidrug resistance is antimicrobial resistance shown by a species of microorganism to multiple antimicrobial drugs. Such microorganism mostly result into therapeutic failure and even spread the resistance among other species of bacteria by horizontal gene transfer. In our study highest Multidrug resistances found (60%) in Gram Positive isolates while among Gram Negative (41%) and Fungi (12%).

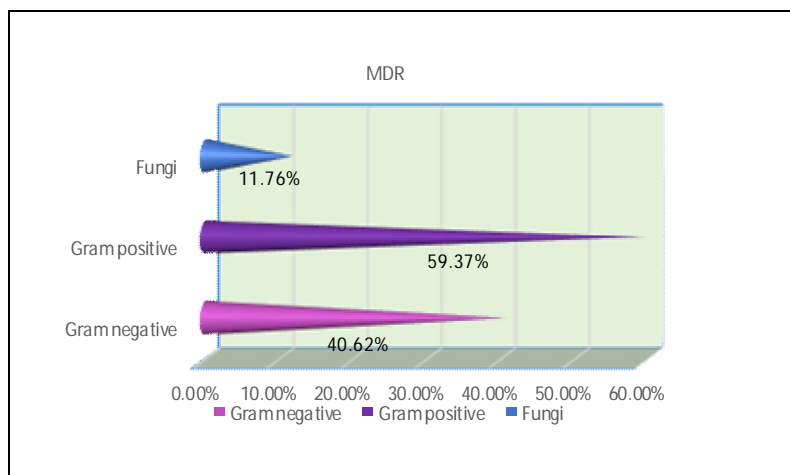


Fig.10 Multidrug Resistance among the clinical isolates

H. Determination of ESBL (Extended-Spectrum Beta-Lactamases) Producers

ESBL is enzyme that confer resistances to most beta-lactamase antibiotics. ESBL producers are most commonly associated with therapeutic failure and therefore poor outcome of infection. ESBL producing organisms have important therapeutic and clinical ramifications for patients. It destroy cephalosporins, main hospital antibiotics, given as first-line agents to many severely ill patients. And all gram negative isolates were tested for ESBL production.

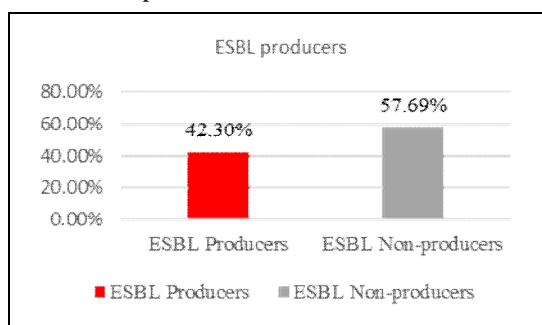


Fig.11 Graphical representation of ESBL producer among isolates.

In present study emergence of ESBL was of 42.03% while in contrast to our study K Manasa saraswathy et al., (2017) reported 68.8%.

I. Detection of Oxacillin-Resistant *Staphylococcus aureus* (ORSA)

Oxacillin is a penicillinase-resistant β -lactam. It is similar to methicillin, but has replaced methicillin in clinical use. Oxacillin is used to treat many different types of infections caused by *Staphylococcus* (also called "staph" infection). An organism exhibiting this type of resistance is referred to as Oxacillin Resistant *S. aureus* (ORSA). In present study 10 isolates (15.62%) were observed as Oxacillin resistance *staphylococcus aureus* while in contrast to our study to S. Rajesh et al., (2017) reported (28 %) ORSA.

J. Detection of Inducible Clindamycin Resistance (D-Test)

We performed D-test for gram-positive isolates against their sensitivity towards clindamycin and to check if there is a macrolid-resistant subpopulation of bacteria present.

Result of D-test is represented in table1:

Susceptibility Pattern (phenotype)	No. of isolates	Percentage (%)
ERY-S,CL-S	6	15.78%
ERY-R,CL-R (constitutive, MLS _B)	4	10.52%
ERY-R,CL-S (D -test positive,iMLS _B)	8	21.05%
ERY-R,CL-S (D-test negative, MS)	20	52.63%

(ERY=Erythromycin, CL=Clindamycin, R=Resistant, S=Sensitive, Constitutive MLS_B= Constitutive MLS_B phenotype, iMLS_B=Inducible MLS_B phenotype, MS=MS phenotype,

K. Detection of Biofilm Forming Capabilities of Isolates

A Biofilm forming microorganisms are a group of microorganisms in which cells stick to each other on a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also referred to as slime (although not everything described as slime is a biofilm), is a polymeric conglomeration generally composed of extracellular DNA, polysaccharides and proteins. All isolates were test for their biofilm production capacity by tube method.

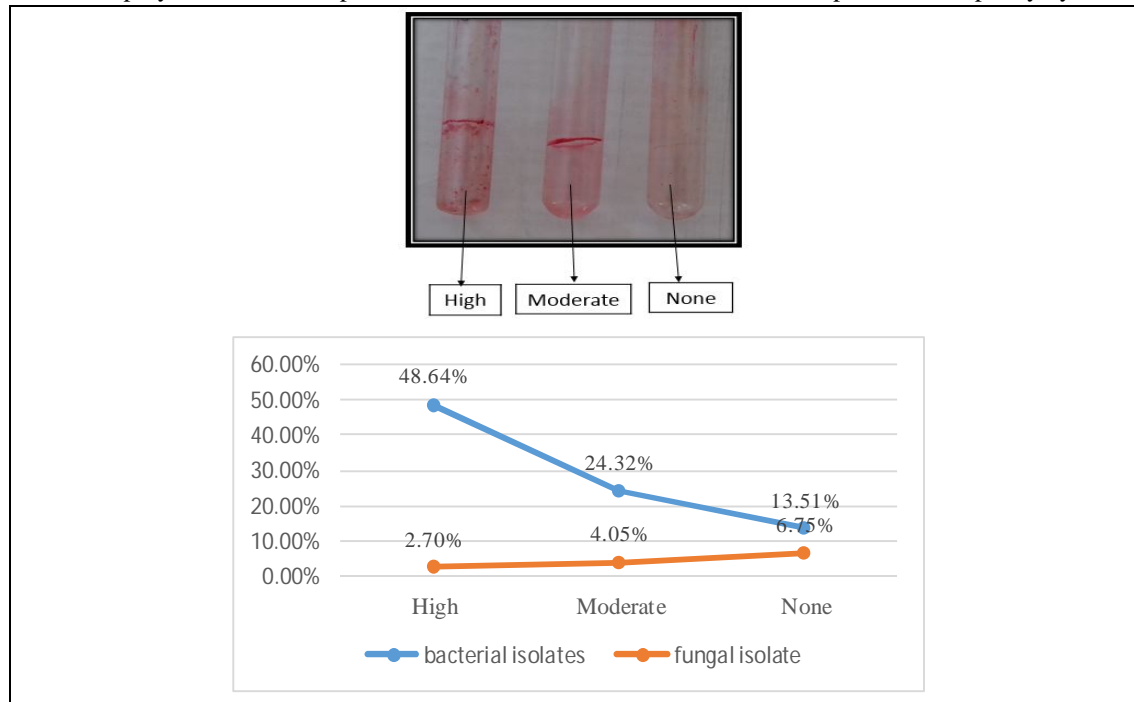


Fig.12 Biofilm formation by Tube method

We reported 56.25% bacterial isolates & 13.51% from fungal isolates as strongly positive results.

L. Biostatistical analysis by SPSS

Statistical analysis was performed by using IBM SPSS statistics 20.0. The analysis of level of significance (p value) for eye infection is done by calculating Chi square test.

Table.2 Results of biostatistical analysis

Sex	Total cases %	Number of positive %	Chi square test	p-value
Male	32 (37.64%)	29 (90.62%)	4.8111	0.028277
Female	53 (62.35%)	45 (84.90%)		

In the present study the chi-square statistic is 4.8111. The p -value is 0.028277. This result is significant at $p < 0.05$. In accordance to our study Anteneh amsalu et al., 2015 the chi-square statistic was 1.154. The p -value is 0.0283. Eye infections left untreated can lead to further complications, such as vision loss. Its prevention includes follow up is required and probiotic supplements are most adequately studied at these point.

M. Molecular Identification by 16S rRNA

In Partial Molecular Identification of the isolate S10 was compared for homology sequence contained within large database using BLAST tool of NCBI. Partial sequence of the isolate showed 100% identity with 16S rRNA partial sequence of *Staphylococcus capitis* strain and identified as *Staphylococcus capitis*. The partial sequence of 16S rRNA of isolate S10 was deposited in NCBI database and the (Accession no. is [MK724060](https://www.ncbi.nlm.nih.gov/nuclot/MK724060)). From the antibiotic susceptibility patterns and biofilm production capabilities isolate no. S10 screened for partial sequencing (16S rRNA Sequence) and phylogenetic analysis. It was carried out at Saffron Life science, Udhna, Surat.

IV. CONCLUSIONS

This present study was intended to determine the prevalence of bacterial etiological agents and their virulent role towards various eye infections. We observed the presence of 64 bacterial pathogens and 10 fungal pathogens that were obtained from 85 clinical samples of eye infections from various Eye hospitals of Surat, Gujarat from December 2018 to March 2019.

Amongst all the studied cases of eye infections, most prevalent cases were of conjunctivitis and corneal ulcers. In our study, higher rate of positivity (77.27%) observed is the stinking outcome of present study. Present study also represented the current scenario of an Antimicrobial Susceptibility pattern of eye pathogens associated Gram Positive bacterial isolates show resistance to Linezolid, Cefotaxime, Roxythromycin, and Ampicillin while majority of Gram Negative isolates was found against Ceftizoxime, Tetracycline, and Cefotaxime. Antifungal susceptibility of fungal isolates revealed the higher level of resistance towards antifungal agent Clotrimazole, Fluconazole, Ketoconazole.

Therefore, to prevent the increasing rate of antimicrobial resistance the practice of starting empirical antimicrobial agents should be avoided and the routine identification of bacteria by culture methods and conducting drug susceptibility testing should be practiced in addition to that direct Gram staining and wet mounting, as a routine diagnostic procedure especially in the centres where ophthalmologists have access to microbiology lab facilities. Improper selection of antibiotics, inadequate dosing and poor compliance to therapy may play an important role in increasing resistance. Changes in bacterial resistance patterns have been a major problem in the effective management of ocular infections, better access to effective and safe topical antibiotics has been cited as the primary factor in improving patients outcomes and quality of life. Early access to diagnosis and appropriate treatment and better patient health education can prevent the ocular morbidity and mortality.

V. ACKNOWLEDGMENT

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