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Screening and biochemical characterization of beta hemolytic strains of *Staphylococcus aureus* from Conjuctivitis infections

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Abstract

Background: Conjunctivitis is one of the most common infections observed in hospitals. Children under the age of seven are at the highest risk for diagnosis, with the most notable incidence occurring between the ages of zero and four. **Objective:** The present study was aimed to identify pathogenic strains of *Staphylococcus aureus* by biochemical tests. **Methodology:** Samples of eye infection were collected from Fatima Memorial Hospital, Lahore. After collection, Samples were spread on Mannitol salt agar (MSA) to get pure culture of *S. aureus.* After isolating pure bacterial strains, the pathogenicity of these strains was assessed using a blood agar test. The strains that exhibited beta hemolysis were selected, and their susceptibility to various antibiotics was evaluated. **Results:** Results showed that all used antibiotics were considerably effective against the pathogenic isolated bacteria. Five strains displayed beta hemolysis, suggesting the existence of pathogenic strains of *Staphylococcus aureus*, known to be associated with Conjunctivitis. Zones of inhibition (ZOI) were noted in response to various antibiotics. **Conclusion:** Effective and accessible treatment could enhance public health worldwide. Easily obtainable treatments may result in improved health outcomes, especially in areas where eye infections are common and access to advanced medical care is restricted. The antibiotic sensitivity test indicated that *S. aureus* is a multidrug-resistant pathogen, with only a small percentage of antibiotics proving effective against it.

Keywords: Conjunctivitis, *Staphylococcus aureus*, Mannitol salt agar, Beta Hemolysis, Antibiotic resistant

Introduction:

The eye is the most delicate organ in the human body. Moreover, a unique microbiota resides on the outermost layer of the eye, inhibiting the growth of pathogens and offering protection without causing discomfort. These natural and adaptive defense mechanisms, in conjunction with the microbiota, serve to physically save the eyes, along with ophthalmic barriers such as the eyelids and tear film, to prevent the growth of potentially harmful organisms under typical physiological conditions [I, II]. Most bacterial infections leading to conjunctivitis originate from the body's natural flora [III, IV]. To effectively diagnose the infection and determine the most appropriate treatment, it is essential to assess it from morphological, pathological, and molecular perspectives [V]. External ocular infections,

including keratitis, dacryocystitis, blepharitis, and conjunctivitis, are frequently caused by bacterial agents. These conditions contribute to rising rates of illness and blindness globally.

The conjunctiva refers to the clear, moisture-producing mucous membrane that encases the outer surface of the eye [VI]. Conjunctivitis is marked by symptoms such as swelling of the blood vessels, eye discharge, discomfort and inflammation of the conjunctival tissue. It can present as either acute or chronic and may be contagious or non-contagious [VII]. Conjunctivitis is commonly triggered by bacterial infections. The responsible pathogens consist of *Moraxella* spp., *Haemophilus* spp., *Streptococcus pneumoniae* and *Staphylococcus* spp. and Children are more susceptible to infections caused by Streptococcus and Haemophilus compared to adults [VIII]. In adults, the main causes are *Staphylococcal* species, particularly *S. aureus*, along with *Haemophilus influenzae* and *S. pneumoniae* [IX]. In contrast, *S. pneumoniae, H. influenzae,* and *Moraxella catarrhalis* are more commonly responsible for infection in children [X].

Neisseria gonorrhoeae, *Chlamydia trachomatis* and *Corynebacterium diphtheriae* are additional bacterial offenders. In newborns and sexually active adults, bacterial conjunctivitis is predominantly caused by *Neisseria gonorrhoeae* [XI]. In cases of chronic bacterial conjunctivitis, the ocular symptoms and signs persist for a minimum of four weeks. Conjunctival hyperemia and discharge are generally mild to moderate. The organisms most identified are coagulase-positive and coagulase-negative *staphylococci* [XII]. Conjunctivitis (red eye or pinkish) has many different symptoms like redness of the eyes, eye's pain, blurring vision, light sensitivity watery eyes. Pinkish eyes, tears, pain and itching are the most well-known symptoms of eye infections and the inflammation of surface of eye is most common [XIII]. Acute conjunctivitis is typically characterized by symptoms lasting less than 3 to 4 weeks. There are noted seasonal patterns; bacterial conjunctivitis tends to peak from December to April, while viral conjunctivitis is most prevalent in the summer. Allergic conjunctivitis, on the other hand, is more commonly observed in the spring and summer months [XIV, XV].

Methodology:

Samples of conjunctivitis eye infections were collected from Fatima Memorial Hospital (FMH), Shadman Lahore by using sterilized culture swaps. These samples were transferred directly to the microbiology research laboratory of Zoology department in Government College University, Lahore for further study.

To isolate bacterial strains of ***Staphylococcus aureus***, Mannitol salt agar (MSA) specifically designed for its growth was prepared. Following sterilization, the media was allowed to cool down to 40-45 ºC temperature in sterilized laminar airflow cabinet and then media was poured into petri plates. The media was then allowed to solidify in the plates. Once solidified, collected samples were spread onto the media plates by using culture sticks. After the spreading process, the plates were inverted, covered with aluminum foil, and incubated overnight at 37°C. After the incubation time, the growth of ***Staphylococcus aureus*** was checked on the specific media.

After getting the growth of bacteria on Mannitol salt agar (MSA), 2.8 % nutrient agar media was prepared. The media was sterilized by using autoclave at 121 ºC temperature and 15 psi pressure for 60 minutes. The sterilized medium was allowed to cool down and then poured into plates under sterilized laminar air flow, allowing it to solidify. Once solidified, colonies from the specific media plates were streaked onto nutrient agar plates by using an inoculating loop and incubated at 37°C for 24 hours. The growth of bacteria was checked on the nutrient agar [XVI] (Figure 1).

Various morphological and biochemical tests were subjected to ensure the presence of ***Staphylococcus aureus*** [XVII].

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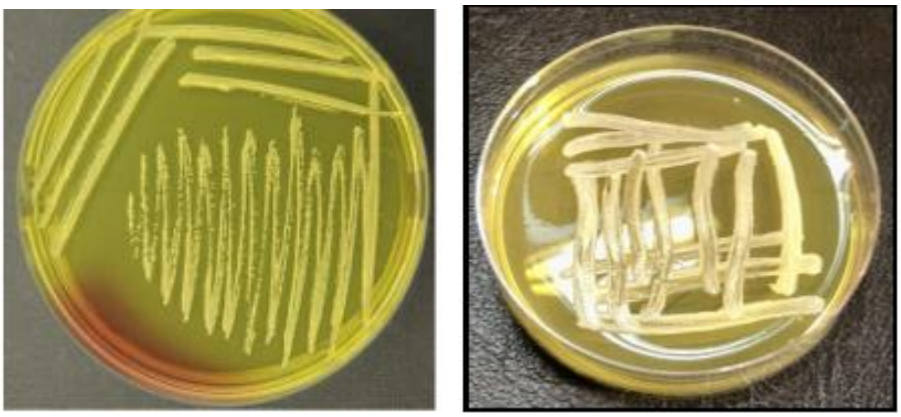

Figure 1: Growth of *S. aureus* on MSA

Blood Agar Test is used to evaluate the pathogenicity of different bacterial strains [XVIII]. Once the blood agar media was prepared, it was poured into plates, and colonies from nutrient agar plates were streaked onto the blood agar. After streaking, the plates were inverted, covered with aluminum foil, and placed into incubator overnight at 37°C. After incubation time, the pathogenicity of the bacteria was checked to determine whether the strains exhibited alpha, beta, or gamma hemolysis. A clear zone surrounding a bacterial colony shows beta hemolysis, which signifies a positive result. In compare, a greenish zone indicates alpha hemolysis and the absence of zones indicates gamma hemolysis, means no hemolytic activity.
The results indicated that the bacteria exhibited a slightly spherical smooth texture. Moreover, these colonies demonstrated β-hemolysis when cultivated on blood agar (Figure 2).

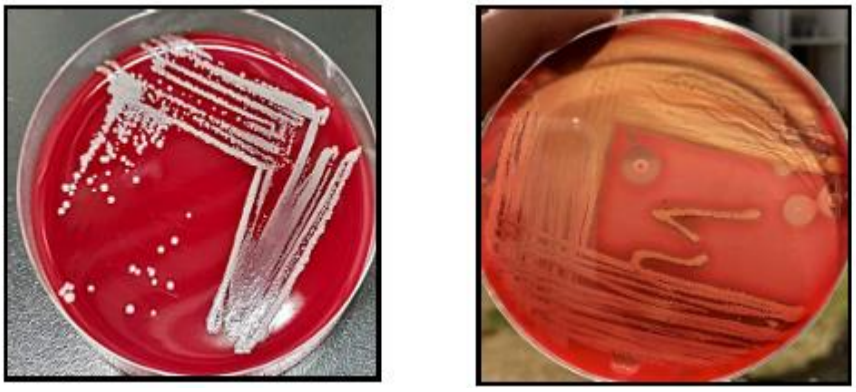

Figure 2: Growth of *S. aureus* on blood agar at 37 °C for 24 hrs

Isolated bacteria produced yellow (golden) colonies as a result of mannitol fermentation, which alters the phenol red indicator to a golden while exhibiting resistance to high salt concentrations present in Mannitol Salt Agar (MSA), a selective medium. These characteristics are indicative of *Staphylococcus aureus* [XIX, XX].

This differential culture medium is specifically designed for S. aureus, inhibiting the growth of other bacterial species [XXI]. The growth of S. aureus on Chromagar TM a medium specifically formulated for its detection and isolation, was employed to conduct a more in-depth analysis of these isolated S. aureus [XXII] (Figure 3).

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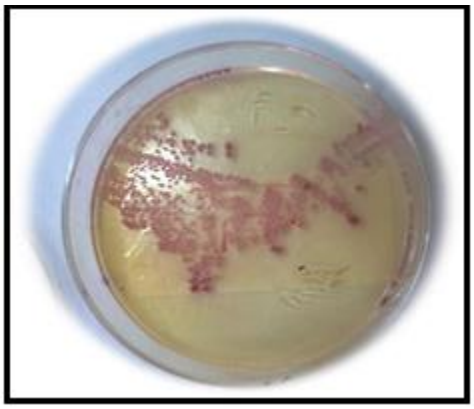

Figure 3: Colonies of *Staphylococcus aureus* on Chrmoagar

To preserve pathogenic bacterial strains, Glycerol stocks were prepared for future use. 2.8g of nutrient broth was prepared in a flask, which was then sealed with a cotton plug and aluminum foil, autoclaved for 1 hour at 121°C and 15 psi and allowed it to cool. After this, the nutrient broth was poured into test tubes and isolated colonies of pathogenic bacteria were inoculated into the tubes (one test tube for each bacterial colony) with a sterilized red-hot inoculation loop. The test tubes were sealed with cotton and incubated overnight at 37°C. The presence of turbidity in the nutrient broth indicated the growth of bacteria. 200µl sterilized glycerol was poured into an autoclaved Eppendorf tube. Subsequently, 800µl of the broth culture was added to the same Eppendorf tube, mixed using a vortex, and stored at -20 °C for future use.

Biochemical tests for Isolated Bacteria:

Morphological Characteristics:

After incubation, growth was observed on both MSA and NA plates. The colonies exhibited various types. Colonies from the NA and MSA plates were chosen for further identification and were isolated using a loop [XXIII] (Figure 4).

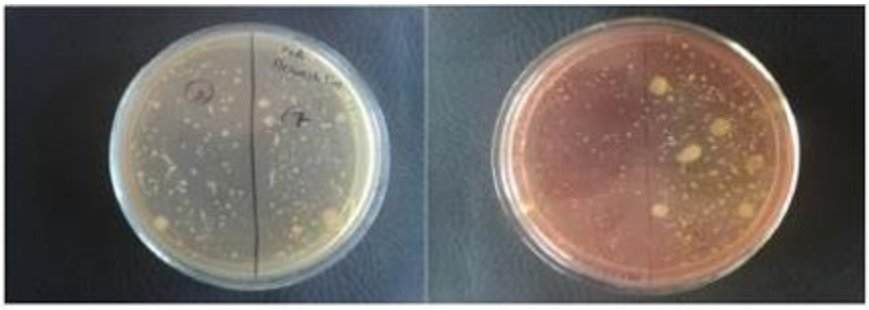

Figure 4: MSA and NA plates showing growth after incubation

Gram's staining:

The examination of specimens diagnosed clinically was conducted using a microscope. The Gram staining revealed the characteristics of *Staphylococci* spp., which exhibited a Gram-positive reaction, were non-spore-forming, and appeared as irregular clusters with a cocci shape, as illustrated in [XXIV] (Figure 5).

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Gram staining was conducted to distinguish between gram- negative positive and gram- positive bacteria. The bacterial morphology was determined through the gram staining process.

To perform the gram staining, a glass slide was prepared with a thin layer of a pure bacterial culture. Once the smear was formed, the slide was allowed to air dry and was then fixed by passing it through a flame. After fixation process, crystal violet stain was applied to the sample and allowed to sit for a few seconds and then washed with water. After washing step, iodine solution was applied on slide and left for a few seconds. Alcohol is used for decolorization of smear and then safranin stain was applied for a few seconds. The slide was then rinsed with water. After rinsing, blotting paper was used to dry the slide, which was examined under a microscope [XXV]

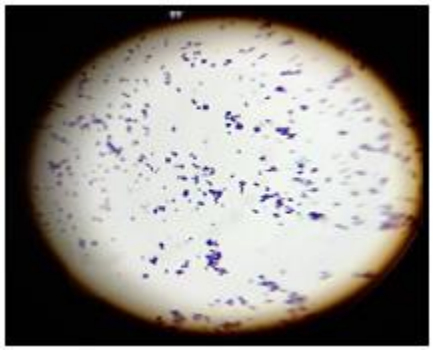

Figure 5: Microscopic examination of *Staphylococci* aureus isolates

MacConkey Agar test:

To distinguish between gram-positive and gram-negative bacteria, MacConkey agar media was utilized. The plates of media were prepared after sterilization and allowed to cool down. Once the media had settled in the plates, the isolated bacteria were streaked onto the media and incubated overnight at 37°C. After the incubation period, results were made to determine whether lactose fermentation had occurred or not [XXVI]

Eosine Methylene blue agar (EMB Agar):

EMB media serves as both differential and selective media for bacterial cultures. It effectively prevents the growth of gram-positive bacteria. The EMB media was prepared using distilled water and sterilized the media using autoclave. Eosine Methylene blue agar media plates were prepared after sterilization and isolated bacteria were streaked onto them. *Staphylococcus aureus* growth is inhibited by EMB Agar and it indicates the presence of *S. aureus* [XXVII].

Endospore staining:

Endospore staining was conducted to differentiate between spore and non-spore-forming bacterial strains. To perform the endospore staining, a thin layer of the bacterial strain was prepared and fixed using a spirit lamp. Once fixation was complete, the smear was covered with blotting paper and placed over boiling steam, where malachite green stain was applied to the smear for a duration of 20 minutes. Allow the slide to cool down. After taking the strips off the slide, it was drained over water and stained with safranin for 2 minutes before being washed under running water. The slide was then dried using blotting paper, and observed under a microscope [XXIX].

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Catalase test:

The catalase test was conducted to determine ability of bacterial strains to break down hydrogen peroxide (H2O2) or not. During this procedure, the isolated bacteria was placed on a slide, and a drop of H2O2 was applied to observe the presence of bubbling. A single colony from the nutrient agar medium is picked and placed onto a slide with hydrogen peroxide, then observed for 1-2 minutes. The formation of bubbles indicates the presence of *Staphylococcus aureus* [XXX, XXXI, XXXII] (Figure 6).

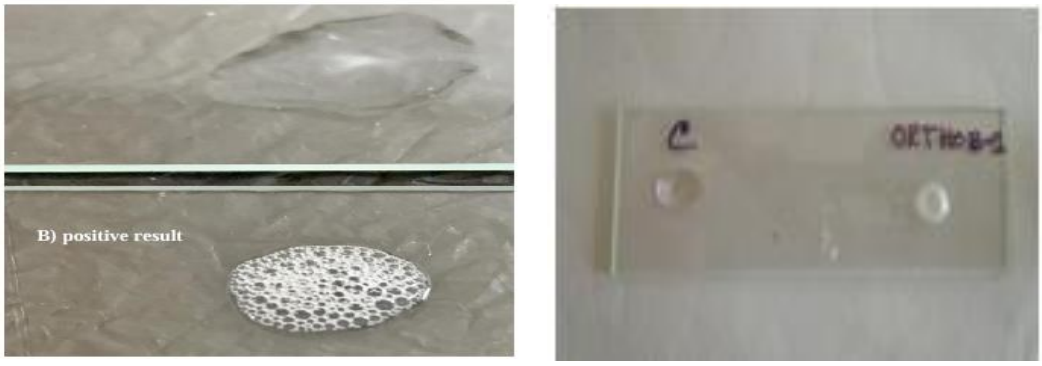

Figure 6: Catalase test for *S. aureus*

Oxidase test:

A piece of filter paper free from heavy metals was taken and placed on a clean Petri dish and three drops of newly prepared oxidase reagent were added to it. Using a red-hot loop, a colony of the isolated bacteria was picked and make smeared onto the filter paper. The absence of any color change indicates a negative result, indicated the presence of *Staphylococcus aureus* [XXX, XXXI, XXXII] (Figure 7).

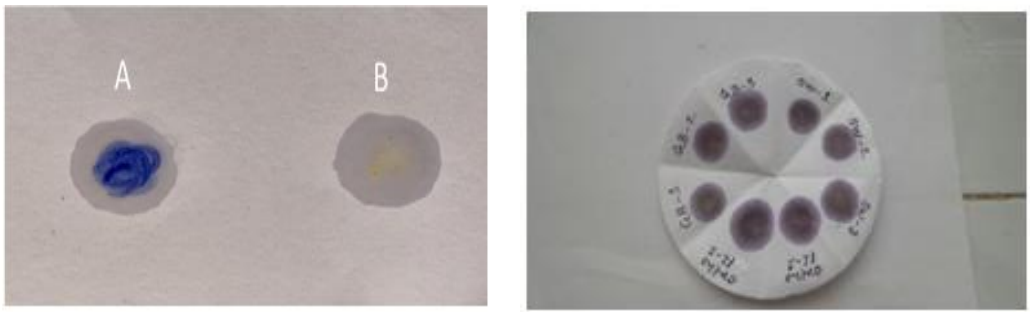

Figure 7: Oxidase test for *S. aureus*; A) Positive result B) Negative result

Coagulase test:

The coagulase test was conducted on the isolated bacteria to identify the presence of *S. aureus*, indicating that it is the widespread microorganism. To evaluate whether the coagulase enzyme is bound or free, a suspension of 0.5 ml of bacterial colony is combined with 0.5 ml of prepared human plasma and incubated at 37°C. The plasma is then periodically examined for fibrin formation and coagulase clot development over a four-hour period. A positive result, indicative of *Staphylococcus aureus* strains, is determined by the presence of a blood clot. Conversely, a lack of clot formation after 24 hours of incubation is considered a negative result. [XXXIII, XXXIV, XXXV, XXXVI] (Figure 8).

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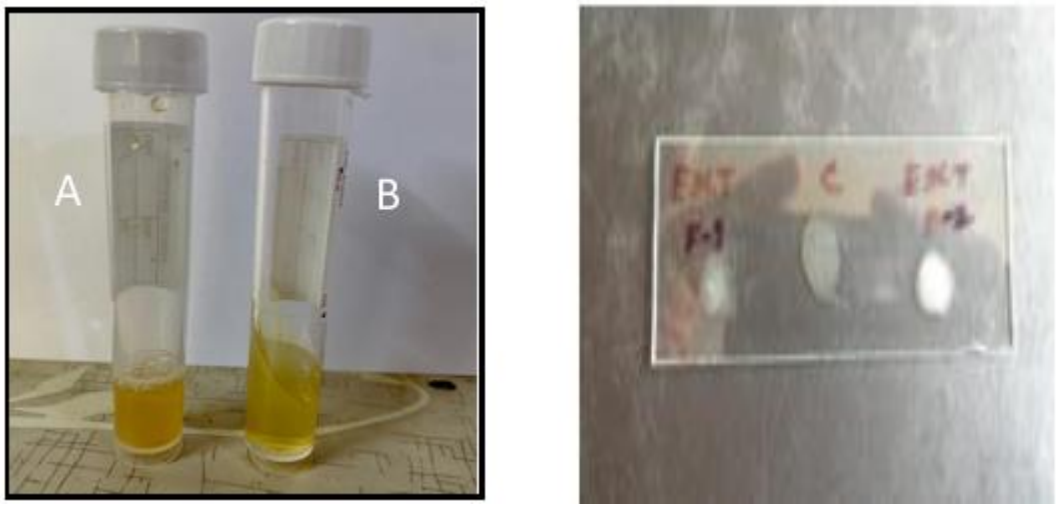

Figure 8: Coagulase test for *S. aureus.* A) Negative result B) positive result

Congo Red test:

The Congo Red test was conducted to identify the formation of biofilm by *S. aureus*. While this test may not be the most sensitive method for assessing biofilm production, it remains a reliable choice due to its adequate sensitivity and specificity. A concentrated solution of Congo Red was prepared and autoclaved. The solution was added to the medium once the agar cooled to 55°C before being poured into petri dishes. The bacterial isolates were inoculated and then incubated of 24 to 48 hours at 35°C.The presence of brown and black colonies with a dry and crystalline texture showed the formation of biofilm (positive result), whereas colonies that appeared red or dark red were classified as non-biofilm productive bacteria (negative result) [XXXVII, XXXVIII].

Antibiotic Activity of antibiotics against Isolated Bacterial Strains:

To evaluate the efficacy of antibiotics, six antibiotic solutions were prepared by dissolving 5g of each antibiotic in 25ml of distilled water. The antibiotics were Azithromycin, Gentamycin, Ceftazidime, Droncef, Vinjec and Ciprofloxacin. The well diffusion method was used to evaluate the antibacterial activity of these antibiotics. Nutrient agar solution was prepared in a flask of 250 ml, which was sealed with cotton and aluminum foil and then autoclaved at 121 °C and 15 psi for 1 hr. After autoclaving, the nutrient agar was poured into six petri dishes and allowed for solidification. Once solidified, in each plate five wells were formed using an autoclaved yellow tip. Bacterial strains (1, 2, 3, 4, 5) were evenly spread across the agar using a red-hot inoculating loop and a glass spreader. Distilled water (50µl, as a negative control) and the six antibiotic solutions were added to the wells using autoclaved blue tips. The plates were not inverted. After incubating at 37 °C for 24 hours, the effectiveness of the antibiotic against the bacteria was evaluated by measuring the zones of inhibition around the wells using a measuring scale (Figure 9).

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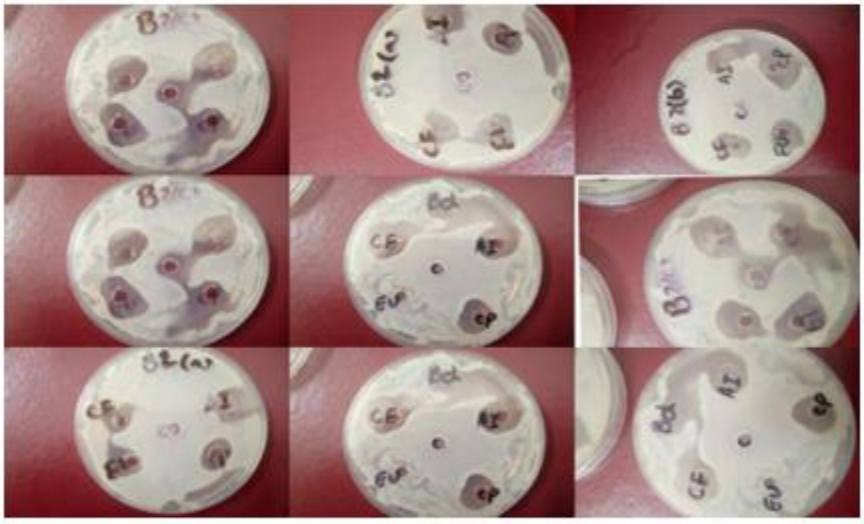

Figure 9: Zones of Inhibition of different antibiotics

Results:

The current research investigates the infection of bacterial conjunctivitis. Five pathogenic strains of bacterial different bacterial strains have been studied among many isolated from conjunctivitis patients

Pathogenicity test for Bacterial Strains:

Strains 1, 2, 3, 4 and 5 bacterial isolates and all showed clear zone. The complete clear zone of β -hemolysis indicated the presence of *S. aureus.*

Effect of antibiotics against isolated bacterial strains by Well Diffusion Method:

Six different types of antibiotics has been used against five isolated strains of bacterial to check their antibacterial activity. ±S.E values of strains 1, 2, 4 and 5 against **Ciprofloxacin** were 0.6±0.316, 1.25±0.19, 1.025±0.05 and 1.02±0.05respectively while strain 5 showed the resistant against Ciprofloxacin. Strain 1 showed the moderate sensitivity against the all antibiotics**. Azithromycin** showed ±S.E values 0.75±0.11, 1.12±0.19, 1.2±0.17 and 0.75±0.19 respectively for strain 1, 2, 3, 4 while strain 3 showed the resistant. For **Ceftazidime**, the ± S.E. values for bacterial strains 1, 3, 4, 5 were 0.575±0.22, 1±0.08, 1±0.08, and 1.02±0.09 respectively indicating that strains 3 and 4 had the same susceptibility level, while strain 3 was resistant. Resistance to **Gentamycin** was found in strain 3 and 5 while strain 1 displayed a value of 0.65±0.23±0 S.E, strain 2 showed 0.61±0.22 and strain 4 showed the value 1.15±0.17 indicating the largest zone of inhibition. For **Droncef**, the ± S.E. values were 0.675±0.17for strain 1, 0.425±0.22 for strain 3, and 1.05±0.05for strain 5, with strain 5 showed the largest zone of inhibition, while strain 2 and 4 were completely resistant. The values of ± S.E. For **Vinjec** across strains 1, 2 and 4 were 0.375±0.17, 0.8±0.25, 12.75±1.314978, and 0.45±0.12respectively. Strain 2 was the most susceptible, while strain 1 was the least and strains 3 and 4 were resistant to Vinjec antibiotic Strains 1, 3, 4 and 5 are more susceptible to ciprofloxacin rather than other five antibiotics.

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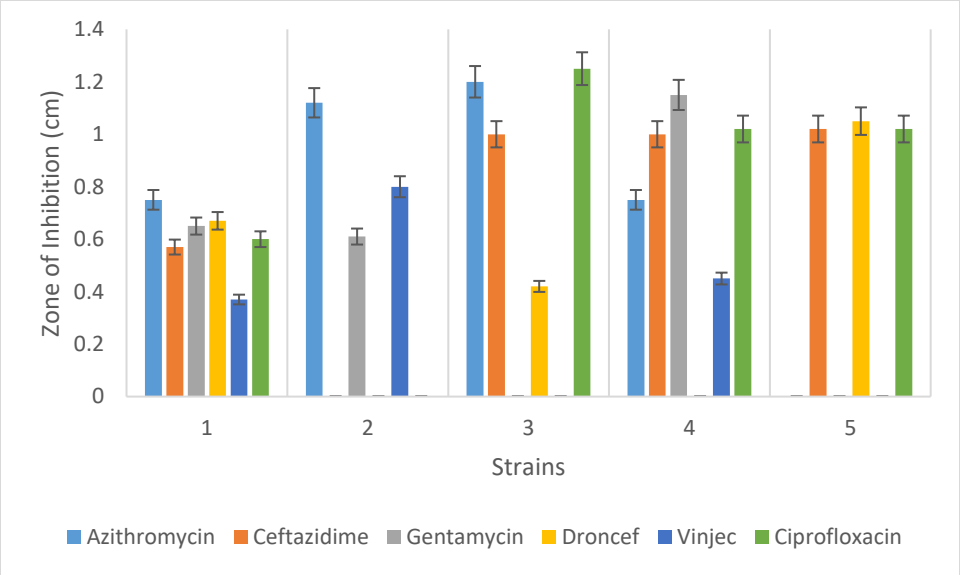

Figure 10: Antibacterial activity against different antibiotics

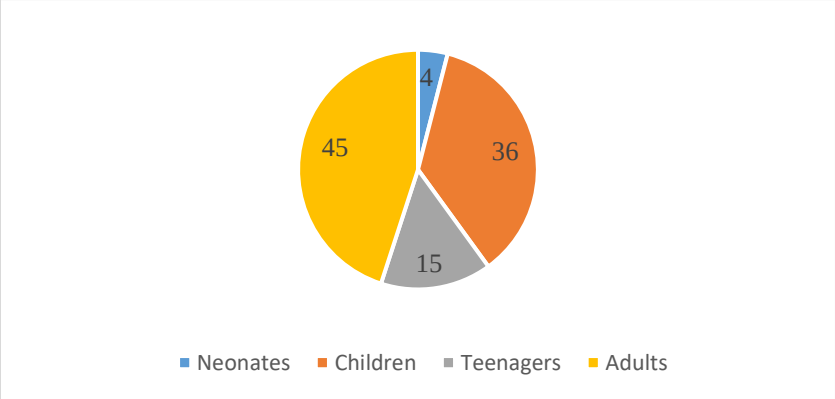

Figure 11: %age of *Staphylococcus aureus* in ophthalmic infections

Table 1: Isolated bacterial strains streaking

Bacterial strains	**Type of Streaking**
Strain 1	Quadrant streaking
Strain 2	Quadrant streaking
Strain 3	Quadrant streaking
Strain 4	Quadrant streaking
Strain 5	Quadrant streaking

Table 2: Hemolysis types of isolated bacterial strains

Isolated bacterial strains	Pathogenicity results	Hemolysis type
Strain 1	+ve	β-hemolysis
Strain 2	+ve	β-hemolysis
Strain 3	+ve	β-hemolysis
Strain 4	+ve	β -hemolysis
Strain 5	+ve	β-hemolysis

Table 3. Antibiotics antibacterial activity by well diffusion method

Strains	Antibiotics Mean (ZOI) (cm)±SED					
	Azithromycin	Ceftazidime	Gentamycin	Droncef	Vinjec	Ciprofloxacin
Strains 1	0.75±0.11	0.575±0.22	0.65±0.23	0.675±0.17	0.375±0.17	0.6±0.316
Strains 2	1.12±0.19	R	0.61±0.22	R	0.8±0.25	R
Strains 3	1.2±0.17	1±0.08	R	0.425±0.22	R	1.25±0.19
Strains 4	0.75±0.19	1±0.08	1.15±0.17	R	0.45±0.12	1.025±0.05
Strains 5	R	1.02±0.09	R	1.05±0.05	R	1.02±0.05

Table 4. Ophthalmic infection %age in different age groups

Age Group	%age of *Staphylococcus* aureus in ophthalmic infections
Neonates	4
Children	36
Teenagers	15
Adults	45

DISCUSSION

The most recognizable symptoms of eye infections involve a pinkish hue in the eye, discomfort, tearing, and itching, often accompanied by inflammation of the eye's surface. Typically, bacterial pathogens target the outer layer of the eye, sparing the internal structures, which remain protected within a sterile environment. Various immunological processes are constantly at work within the interior of the eye [XXXIX]. The conjunctiva employs various defense mechanisms, including immune cells, lysozyme and fibronectin and immunoglobulins that contribute to combating pathogens. A compromised immune system can result in bacterial infections, which can be treated with a variety of antibiotics [XL].

This research focused on eye infections and their sensitivity to different antibiotics used in treatment. By using well diffusion method, antibiotic solutions were utilized on bacterial strains to assess their susceptibility and it was cleared that Ceftazidime demonstrated resistance for bacterial strain 2 while Gentamycin displayed resistance against strain 3 [XLI]. Certain antibiotics have shown different levels of effectiveness against bacterial strains. Tetracycline and chloramphenicol, which are bacteriostatic agents, work by blocking protein synthesis. Although

chloramphenicol is frequently utilized for different bacterial infections in Asian countries, it is banned in the United States because of its harmful effects on bone marrow. Nonetheless, both antibiotics remain viable options for the treatment of eye infections [XLII]. Antibiotic resistance and resistance to bacterial pathogens are interconnected in a similar way. The main factor contributing to this resistance is the inappropriate use of antibiotics for both systemic infections and topical applications. Additional factors, such as prolonged treatment, non-bacterial diseases, and migration, also play a role in the emergence of resistance. Nevertheless, certain antibiotics still demonstrate some effectiveness against bacteria [XLIII].

A One-Way ANOVA test was conducted to compare six solutions of antibiotics. To determine significant differences among these groups and the negative control, a post-hoc analysis using Tukey's test was carried out. All values were stated as standard error mean (SEM) and mean and in the form of graphs.

Ethical Approval

The study was approved by Board of studies (BOS), department of Zoology and Advance Studies and Research Board, Government College University Lahore (REG-ACAD-ASRB/57/24/021). Also approved by ethical committee of institution.

Declarations

All authors listed in paper have made important contributions and there is no conflict of interest among authors.

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