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Prevalence of *Pseudomonas aeruginosa* associated chronic and acute respiratory infections and role of antibiotics as antibacterial agents against isolated pathogens

Short title: *P. aeruginosa* associated respiratory infections

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Abstract

Background: *P. aeruginosa* is the commonest problematic pathogen for acute as well as chronic pulmonary infections in humans. **Objectives:** Screening of pathogenic *P.aeruginosa* from acute through Endotracheal tract secretions and Blood, chronic through Bronchoscopy samples. Identification of pathogenic strains by biochemical testing and virulence assays and Antibiotic resistance sensitivity test was done. **Method:** Blood, sputum and endotracheal samples were collected. Isolation of bacterial strains was done by cetrimide agar media and a number of morphological, biochemical and virulence tests were done for confirmation of the presence of *P. aeruginosa*. Antibiotic sensitivity was done to check the sensitivity of bacterial isolate against different antibiotics. **Results:** The recent study showed the percentile of *Pseudomonas* respiratory infections as, Type II Respiratory Failure 18%, Type I Respiratory Failure 9%, Pneumonia 22%, COPD 9%, Septicemia 15%, Pneumothorax & Emphysema and Co-pulmonale 4%, RDS 5%, Asthma 6%, Chest filial injury and pulmonary odema was 2%. **Conclusion:** Rate of pulmonary infections due to *P. aeruginosa* was high among patients and antibiotic sensitivity test resulted that

P. aeruginosa was a multidrug resistant pathogen and only few percent of antibiotics were effective against this pathogenic bacteria.

Key words: *Pseudomonas aeruginosa*, modern day superbug, quorum sensing, virulence factors, respiratory infections, antibiotic resistance

Introduction:

In immunocompromised people with various infections and diseases *Pseudomonas aeruginosa* is an opportunistic universal pathogen [I]. The *P. aeruginosa* genome as a large genome consists of 6.3 million base pairs (encoding 5567 genes) and is sequenced larger than all other bacterial genomes [II]. There is increasing evidence that the quorum sensing system (QS) is constitutively activated during *P. aeruginosa* infection [III, IV]. *P. aeruginosa* characterized by a number of virulence factors. Endotoxin A, elastase, Pyoverdins, Pyocanin, protease, alginate and most important is the ability of *P. aeruginosa* to form biofilm [V, VI, VII]. In *P. aeruginosa*, three Quorum sensing systems control the virulence factors which are usually known as LuxI/LuxR-type and the last third one is known as *Pseudomonas* quinolone signal system [VIII, IX, X]. Biofilm formation is also controlled by QS of *P. aeruginosa* [XI, XII, XIII, XIV].

High rate of mortality in humans and infants is due to *P. aeruginosa* which cause respiratory distress syndrome (RDS) especially hospital-acquired pneumonia (HAP), community acquired pneumonia (CAP) and Ventilator Associated Pneumonia (VAP) [XV, XVI, XVII]. *P. aeruginosa* is the major cause of VAP due to its ability to form biofilm in instruments such as endotracheal tube etc and VAP has higher prevalence rate in under developed countries having no or limited resources [XVIII, XIX, XX]. Mortality rate is about 70-80% due to VAP caused by *P. aeruginosa* because it is the lethal pathogen which remains in the lungs due to its various virulence factors [XXI, XXII]. About 70,000 to 100,000 humans affected by cystic fibrosis (CF), worldwide. Cystic Fibrosis (CF) is also known as multi-system disease, because respiratory and digestive system mainly affected by CF. Predominantly *P. gaeruginosa* isolated from patients and become the reason of death and high rate of mortality in hospitals [XXIII, XXIV, XXV, XXVI]. *P. aeruginosa* causes severe lethal diseases because it has ability to develop antibiotic resistance. *P.aeruginosa* also form biofilm due to adaptation to various environmental conditions and acquire different genetic abilities for resistance [XXVII]. Antimicrobial drug resistance of *P.aeruginosa* become the global challenge for patient's

suffering from RDS, VAP and CF because still no proper vaccine has been developed for its treatment due to which mortality rate is increasing day by day [XVII, XXVIII].

Methodology

For isolation of bacterial strains (*P.aeruginosa)*, specific media for *P.aeruginosa'*s growth Cetrimide agar media was prepared. After sterilization of media, let the media to be normal at room temperature and pour into petri dishes under laminar air flow cabinet and let the media to solidify in plates. After solidification, spread the samples obtained from patients into media pates. After spreading, invert the plates and cover with aluminum foil and place into incubator at 37ºC for 24 hours. After incubation period, bacterial growth was checked on specific media [XXIX].

After bacterial growth obtained on cetrimide media, nutrient agar media was prepared. Sterilized medium was poured into plates under UV box and let the media to solidify in plates. After solidification, each colony from specific media plates was streaked to nutrient agar media plates and incubated for 24 hours at 37ºC. Bacterial growth was checked on nutrient agar media [XXX]. Different virulence, morphological and biochemical tests were performed to confirm the presence of *P.aeruginosa.*

Pathogenicity Test:

Blood agar media was prepared to check pathogenicity of bacterial strains. After preparation of blood agar media, media was poured into plates and streak the colonies from nutrient agar media plates on to blood agar. After streaking, invert the plates and cover with aluminum foil and place into incubator at 37ºC for 24 hours. After incubation period, bacterial pathogenicity was checked, whether bacterial strains show alpha, beta or gamma hemolysis.

MacConkey Agar test:

To differentiate between gram positive and gram negative bacteria MacConkey agar media was used. After sterilization, media plates were prepare and let them settle down in plates. After settling of media in plates, streak the bacterial isolates onto the media and incubate for 24 hours at 37ºC. After incubation, results was observed whether lactose fermentation occur or not [XXXI].

Eosine Methylene blue agar (EMB Agar):

EMB media is used as both selective as well as differential media for bacteria. EMB media has ability to inhibit the growth of gram positive bacteria. EMB media was prepared in distilled water and sterilized in autoclave. After sterilization EMB media plates were prepared and streaking of bacterial isolates were done. Pinkish colonies showed positive result of presence of *P.aeruginosa* [XXXII].

CLED Agar:

Cysteine lactose electrolyte deficient agar (CLED agar) was used for differentiation. Streaking of bacterial isolates on CLED media plates were done and incubated for 24 hours at 37^{o}C. After incubation period, colonies color were checked whether it was matted green translucent colonies (presence of *P.aeruginosa*) or not [XXXIII].

Gram's and Endospore staining

Gram staining was performed to differentiate gram positive and gram negative bacteria. Bacterial shape was identified by gram staining [XXXIV]. For differentiation spore forming and non-spore forming bacterial strains, endospore staining was performed.

Motility test:

LB agar media with 0.5% glucose, was used for motility test. 1µl of LB broth culture of bacterial strain was added in agar media and incubated for 24 hours at 37^{o}C. After incubation, zones of swim and swarm was observed [XXXV].

Biofilm formation:

The micro titer dish assay is an important tool for studying the initial stages of biofilm formation and it is primarily used for studying bacterial biofilms.

Pseudomonas aeruginosa isolates were cultured in LB media. Dilute the culture 1:100 into freshly prepared LB medium. Pour 100µl of bacterial strain dilution into each well of 96 wells plate. After pouring the bacterial strain into wells, incubate the micro titer plate for overnight at 37^{o}C. After the incubation, shake the liquid and throw it out of the cells. Slowly immerse the plate in water and then take it out and remove water. Repeat the step for twice. 0.1% solution of crystal violet of quantity 125 ul was poured into the wells and incubated for

10to15 minutes at room temperature. Wash the plate with water 3-4 times. Remove excess water by blotting it with tissue paper and let it dry in the air for overnight. Photographs of the results were taken [XXXVI].

Qualtative Assay:

The colonies of Pseudomonas aeruginosa create a yellow-green zone under UV light due to the production of fluorescein. If pyocyanin is also present, it produces a bright green color. To confirm pyocyanin, colorful pigments can be extracted along with chloroform. Most pyocyanin-producing strains also produce fluoroscein, which only produces one color.

King B agar media was prepared and 10ml glycerol was mixed in the media. Sterilized the media. After sterilization, media was poured into the test tubes and streak the bacterial strain for the preparation of slants. Incubate the tubes for overnight at 37^{o}C. Photographs of the results were taken.

Quantative assay:

Pyocyanin toxin (phenazone) was extracted from acid chloroform using a cell-free supernatant. Pyocyanin was measured for its absorption at 520 nm in acidic form [XXXVII].

Rhamnolipid production:

Orcinol method.was used to check the production of rhamnolipid. Bacteria cultures were incubated at 37^{o}C for 125 rpm speed for overnight with shaking. After that, a 1 mL sample was collected and centrifuged. Cell supernatant with 0.5ml ethyl acetate three times was used to extract rhamnolipid. Orcinol reagent fo 1ml was added into sample and incubated for 30 minutes at 80^{o}C. After incubation, cool down the samples at room temperature. Spectrophotometer was used to measure the absorbance at OD522nm [XXXVIII].

Antibiotic Sensitivity test:

Muller-Hinton agar (MH) was used as a medium for antibiotic sensitivity testing. Firstly, 31 grams of MH was added to 1000 milliliters of distilled water, which was then autoclaved at 121 degrees Celsius The media was then placed in disposable petri plates and allowed to dry. Pure culture was used as an inoculum Immerse a cotton swab in the broth culture of a pathogenic bacterium, and streaking was done three times on an MH agar plate. Plates were let them to dry for 5 to 10 minutes. Antibiotic (Impenem, Meropenem, Amikacin, Aztronem, Levofloxacin, Tobramycin, Piperacilin/Tazobactam, Ciprofloxacin, Cefatriaxone, Cefexime, Ceftadizime, Cefaparazone/sulbactum, Ampicillin, Erythromycin, Gentamicin, Moxifloxacin, Ofloxacin) discs were placed

aseptically and then incubated for 24 hours at 37ºC. Zone of inhibitions around the antibiotic discs were observed and measured [XXXIX].

Results

Samples of endotracheal tract secretions and blood were collected from patients of pulmonary problems. Recent studies show that the percentages of Pseudomonas lung infections are as follows: type II respiratory failure 18%, type I respiratory failure 9%, pneumonia 22%, chronic obstructive pulmonary disease 9%, sepsis 15%, pneumothorax, emphysema pulmonary comorbidities 4%, respiratory distress syndrome 5%, asthma 6%, chest pain and pulmonary edema 2%. The results showed that the main lung disease in patients was pneumonia with 22%, followed by type II respiratory failure and sepsis (Figure 1).

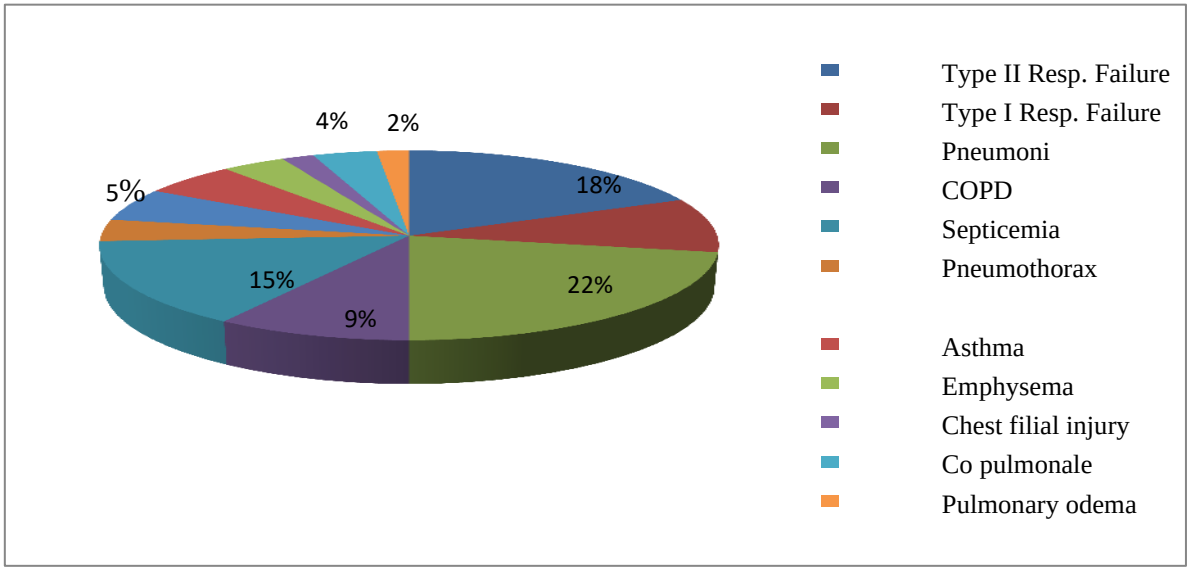

Figure 1: Percentile of Pulmonary infections.

In this study, Gram-negative *P. aeruginosa* isolates from VAP patients tested higher in ETT culture at 57.7% (26/45) compared to 24.4% (11/45) from blood; Gram-positive cocci were 76% higher in blood (34/45) and 42% (19/45) from ETT culture. In a recent data collection, of patients suffering from pneumonia bronchoscopic culture also showed a higher rate of 8/15 (53.3%) (Figures 2, 3 and 4).

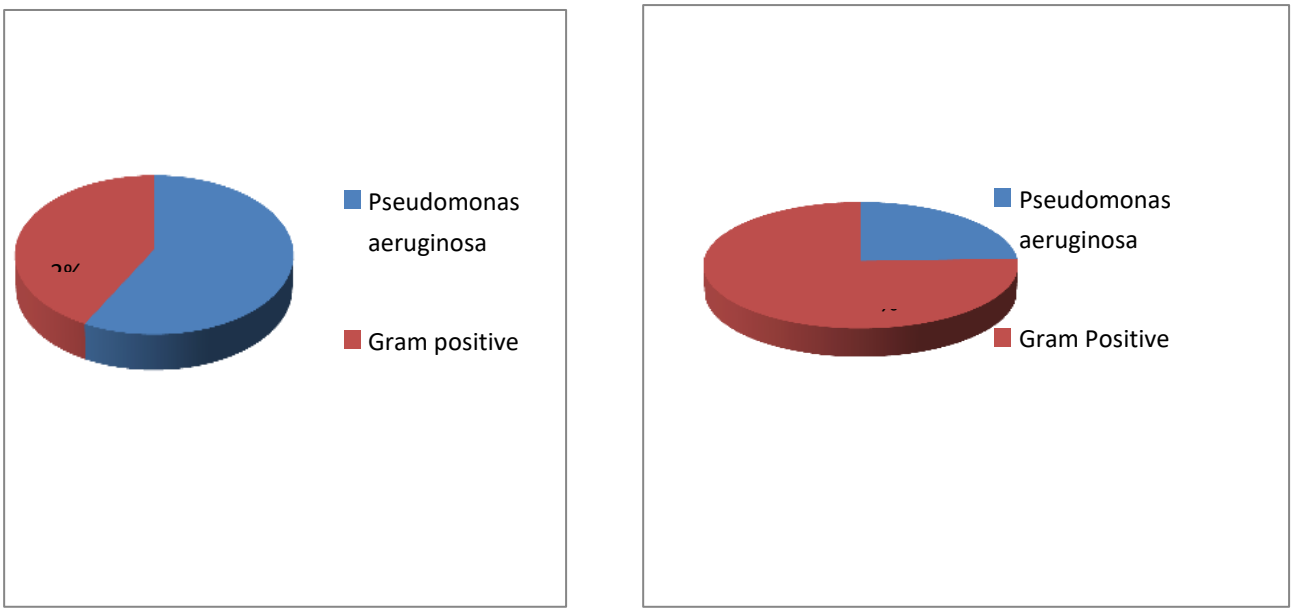

Figure 2: Distribution of *P.aeruginosa* and Gram +ve cocci in VAP ETT culture (left) & *P.aeruginosa* and Gram +ve cocci in VAP blood culture

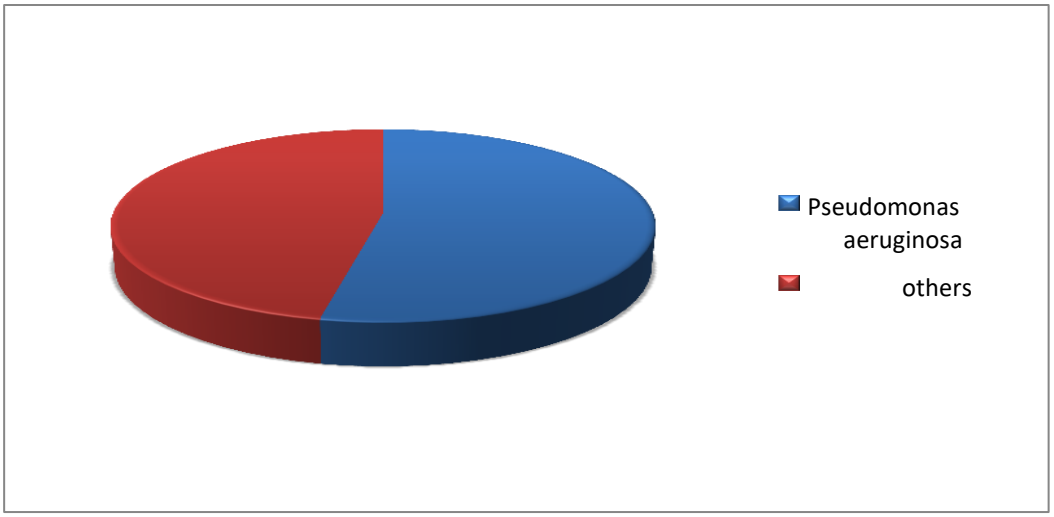

Figure 3: Distribution of *P.aeruginosa* in CF bronchoscopy samples

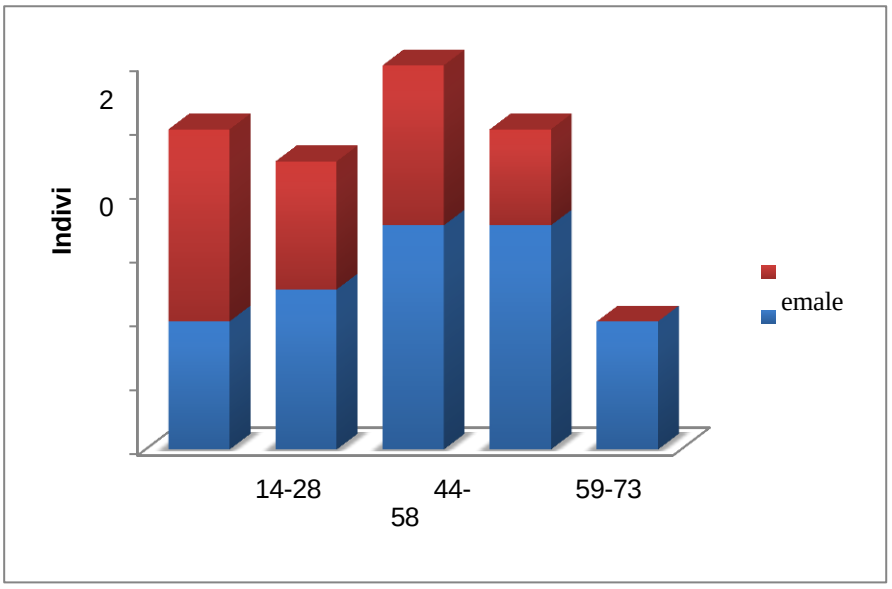

Figure 4: Distribution of pulmonary infection (VAP) Age wise.

Antibiotic Susceptibility testing (Kirby Bauer disc diffusion method):

Analysis of antibody patterns of bacterial isolates using the Kirby-bauer disk diffusion method. The area (zone of inhibition) was measured in millimeters (mm) where bacterial isolates are resistant to antibiotics. All strains are resistant except three which were susceptible to some antibiotics.

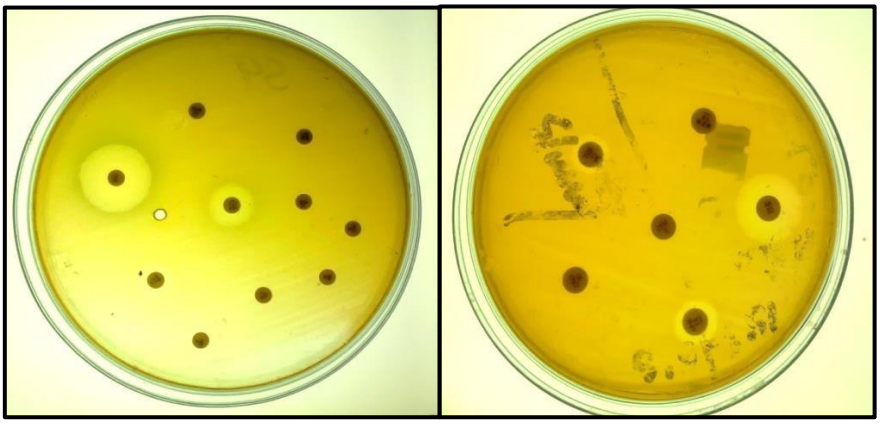

Figure 5: Shows antibiotic sensitivity of *P.aeruginosa*

All isolates were multidrug resistant and MAR (Multiple Antibiotic Resistance) index was at least 0.8. Finally, multidrug resistance of *P. aeruginosa* was investigated in this study. Average of all antibiotic resistance samples belonging to antibiotics used against *Pseudomonas aeruginosa* isolated from samples were calculated as 76% (13/17) resistant and 23.5% (4/17) antibiotic pain protection percentage (Figure 5). The results showed that impenem and meropenem were 11% effective, amikacin was 33.3%, and piperacillin/tazobactam was 22.2%. However, the resistance between IMP

and MEM was 90%. , CES, AMP, E, CN, MXF and OFX were 100% resistant. Almost all of the P. aeruginosa strains tested in this study were pathogenic bacteria (Figure 6).

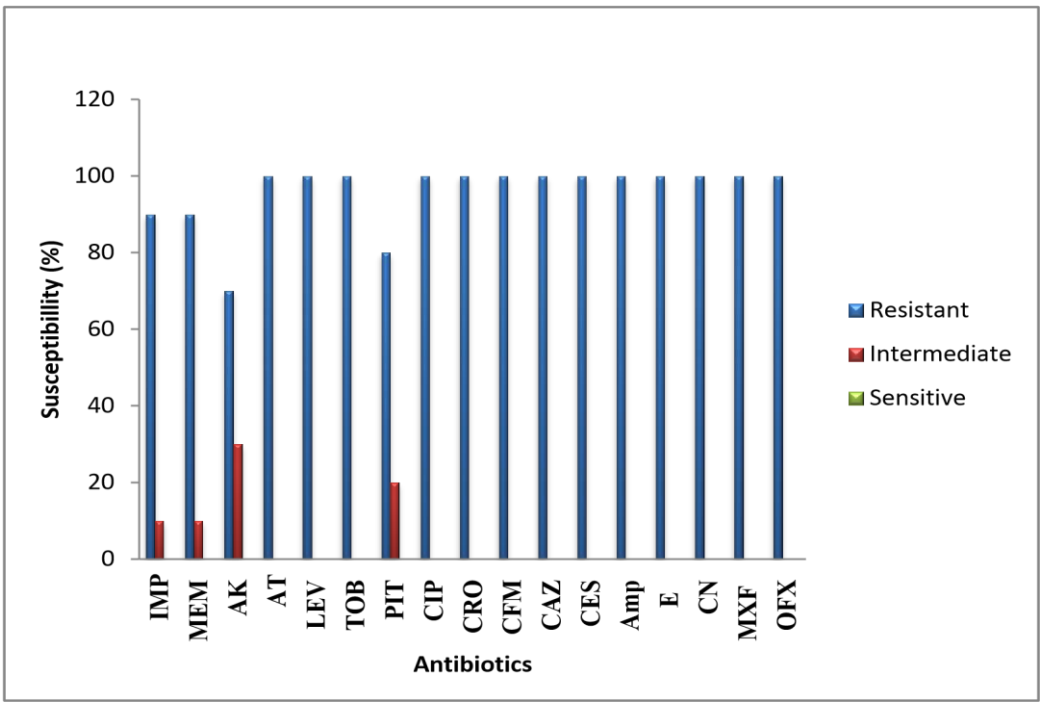

Figure 6: Antibiotic susceptibility pattern of *P.aeruginosa* isolates.

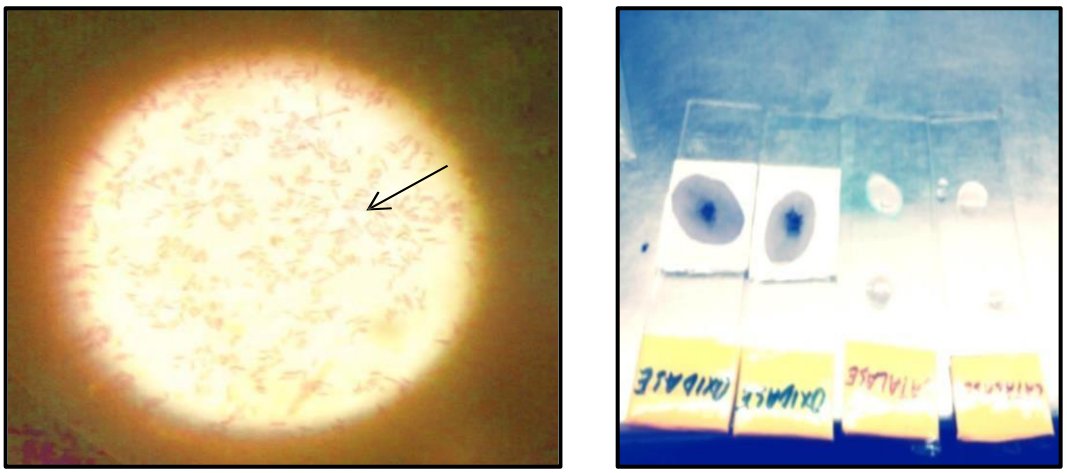

Figure 7: (a) Shows Gram's stain results of *P.aeruginosa* (Gram negative rods of Pink or red colour) (b) Bacterial sp. Catalase and Oxidase positive

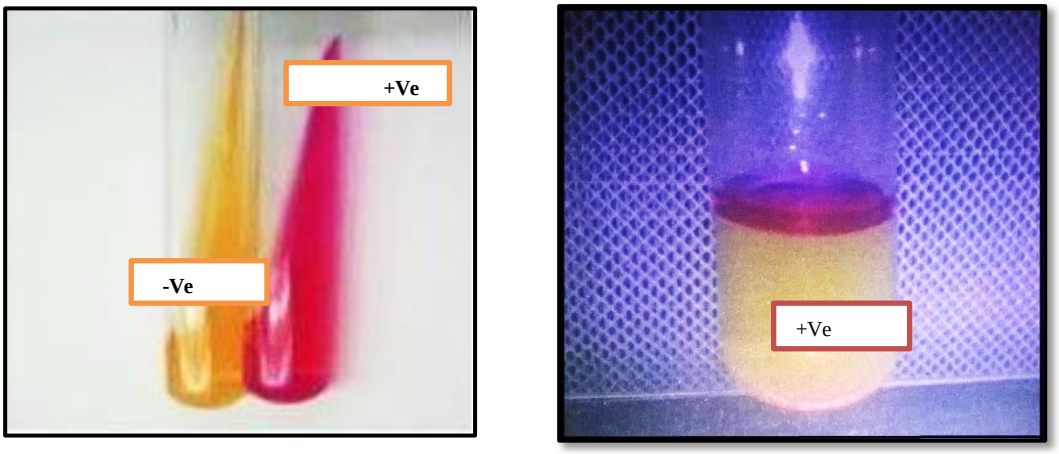

Figure 8: Shows Urease negative results of *P.aeruginosa* (Leftt) (b) shows the positive result of indole test

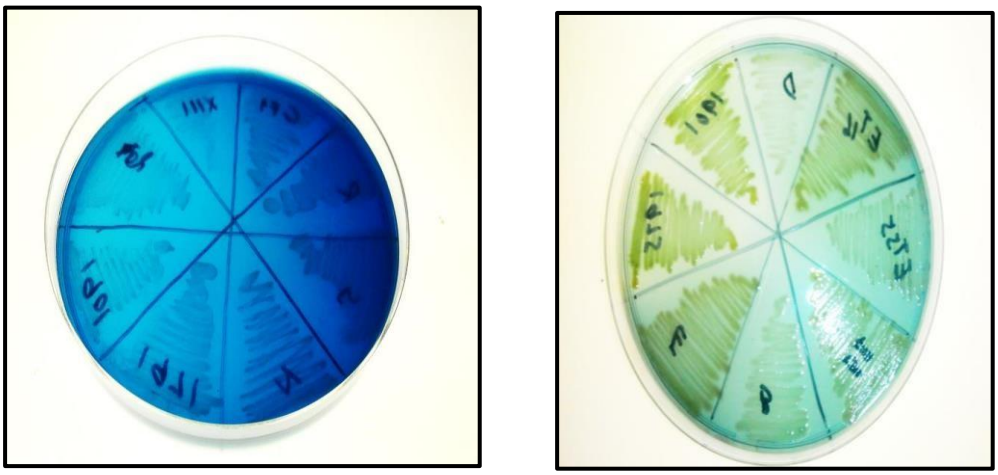

Figure 9: (a) Citrate Positive; change in colour from green forest to deep blue (b) Confirmation of *P.aeruginosa* culture on CLED media; Green translucent Colonies

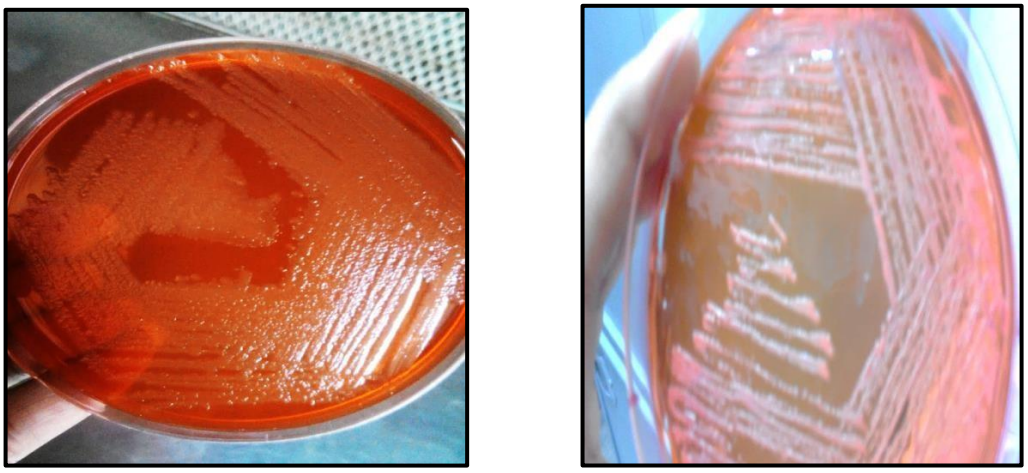

Figure 10: (a) Shows *P.aeruginosa* culture on Mac Conkey agar (nonfermenter) (b) Culture on EMB (Eosin Methylene Blue Agar); Non fermenter pinkish colonies

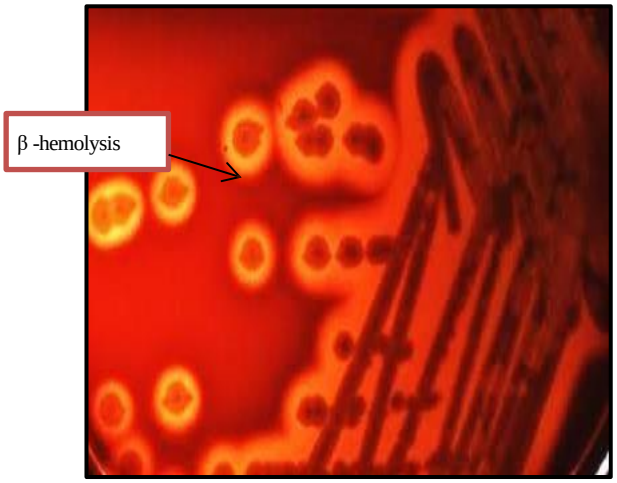

Figure 11: Shows *P.aeruginosa* culture β hemolysis on blood agar

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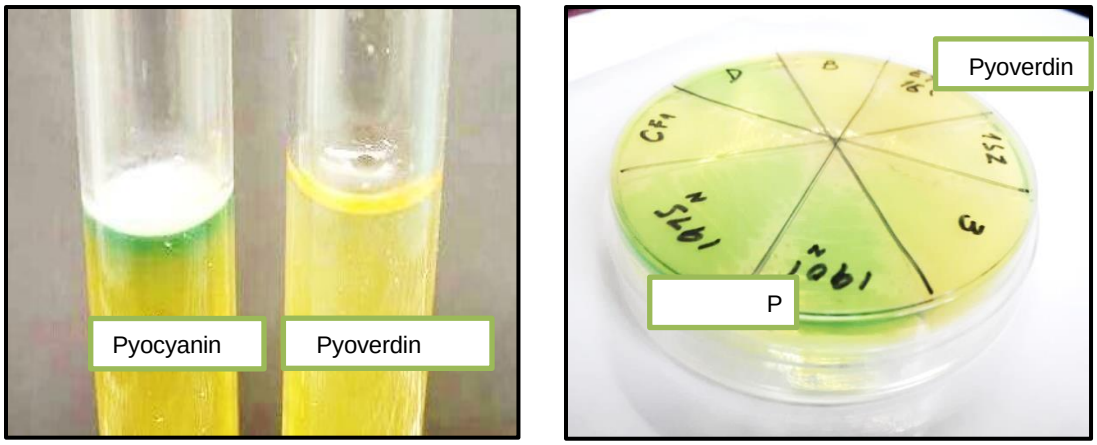

Figure 12: (a) Shows pigment production in LB Broth (Left) Pyocyanin (Right) Pyoverdin (b) Shows pigment characterization on MH agar.

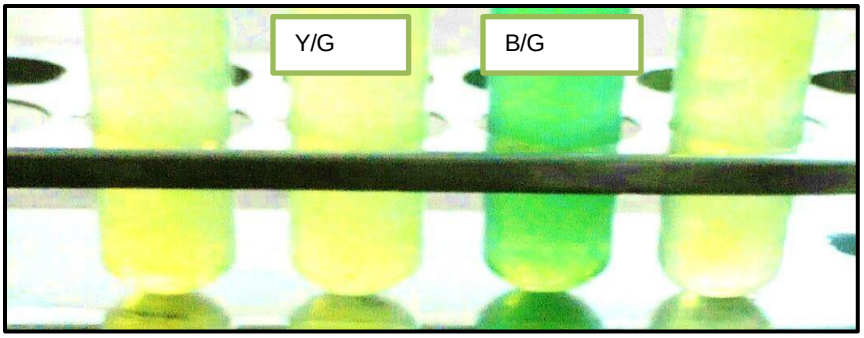

Figure 13: *P.aeruginosa* slants showing yellow green/ blue green pigment characterization.

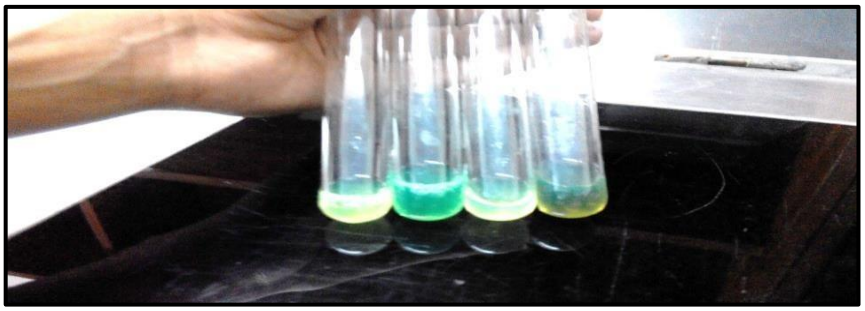

Figure 14: Shows pyocyanin pigment extract.

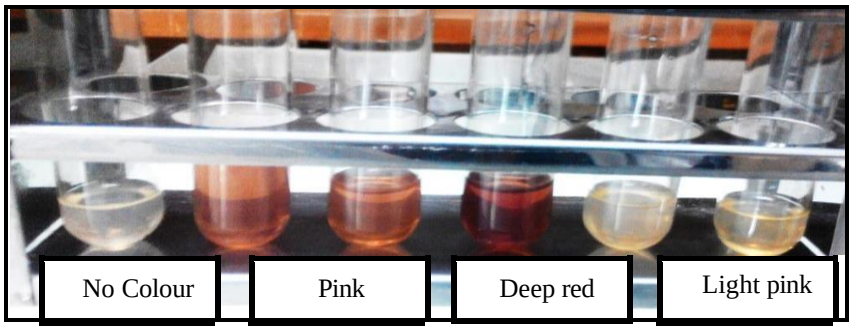

Figure 15: Shows the presence of pyocyanin by colour indication (Deep red/Light pink.

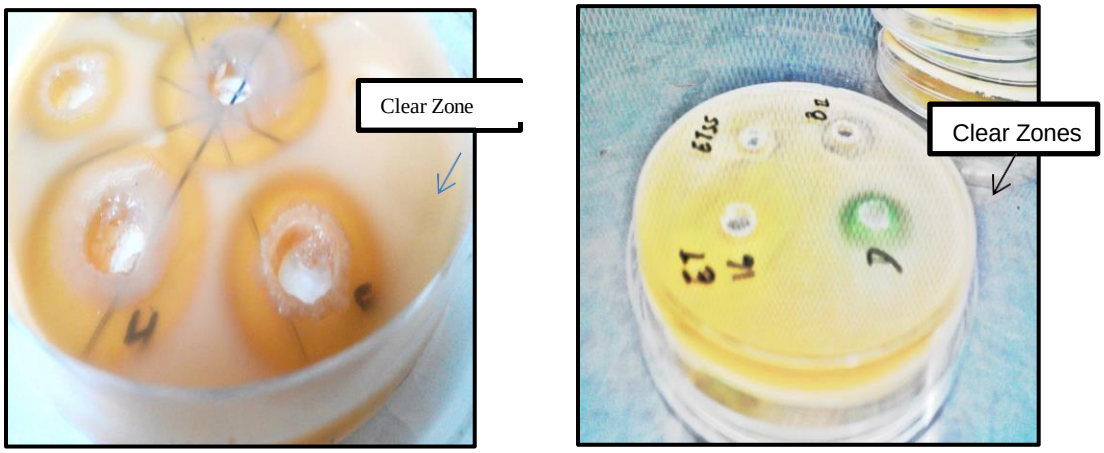

Figure 16: (a) Shows protease test results (Clear zone) (b) Shows Staphlolytic activity of *P.aeruginosa* (clear zones).

Discussion:

Pseudomonas aeruginosa is a Gram-negative rod-like bacterium that can cause infectious diseases and many medical complications. Firstly, species were identified by morphological and physiological tests and then confirmed by bacteriological and antibiotic tests according to CLSI 2007 (Figures 7-16).

Recent studies supported by past studies as all behavioral groups were tested and all P. aeruginosa tests were positive. The MAR index of the isolated Cyclospora assay was 0.6. The minimum MAR index value is less than 0.2, and recent studies show that the MAR index is 0.8, which is more than 0.2,

indicating resistance to the bitterness of many pesticides. 48% of males are infected, while 52% of females are infected. Pseudo Monocystis infections are more common in males, with 56%. (2008) have comparable results. This is due to the differences between the region and the clinic. *Acinetobacter orphii* and most recently *Enterobacter* [XL].

Recent clinical data show that the most common bacteria in VAP and CF are *Pseudomonas aeruginosa*, with 57.7% and 53.3%, respectively. Results vary due to geographical differences. / Piperacillin 20% and meropenem 17%. Songara et al, 2014 found 51% and 38% for gentamicin and amikacin, 55% for ceftazidime, 63% for ciprofloxacin, 56% for levofloxacin, 29% for impenem and 27% for meropenem when Rashid was high. Amikacin 40.4%, Impenem 30.9%, Cefixime 26.19%, Aztronem 21.4% and Gentamicin 16.6% were highly resistant. Recent studies have shown high resistance to impenem and meropenem 90%, amikacin 70%, tazobactam/piperacillin 80%, followed by levofloxacin, tobramycin, ciprofloxacin, ceftriax Pine, ceftazidime, cefoperazone-sulbactam, ampicillin, moxifloxacin, moxifloxacin and ampicillin. All are 100% resistant to lung diseases. The differences in these results are due to specific antibiotics and regional differences [XLI].

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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