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Original Article

Susceptibility Pattern of Pathogenic Bacteria Pseudomonas aeruginosa to various Antibiotics isolated from Post-Surgical Wound of Diabetic Patient at Hayatabad Medical Complex Peshawar

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ABSTRACT

Pseudomonas aeruginosa is a gram-negative rod shape opportunistic bacteria involved in nosocomial infection and resistant to most of the commonly used antibiotics. **Objective:** To find out the antibiotic susceptibly pattern of P. aeruginosa. **Methods:** The current study was carried out on a sample of Diabetics patients having post-operative wounds in Hyatabad medical Complex. The pus sample was screened of pathogen bacteria P. aeruginosa was isolated from most of the sample i.e. 56% while other microorganisms isolated were i.e. E coli, S. aureus, Proteus volugarus. Klebseilla spp coliform, Aclnelobacter spp, Proteus merabillus Provedentia spp. For the identification, different tests were performed including culture growth characteristic Gram staining, biochemical test. Antibiotics susceptibility test where performed utilizing disc diffusion method on Mueller Hinton ager. **Results:** Out of 200 samples, 112 (56%) sample were positive for P. aeruginosa. Infection rate was high in male than female (2:1). The date of the admitted patients in hospital show higher chance of infection as compare of out-patient department (OPD). Among the bacterial isolated 67% where resistant and 31 % were sensitive to cefotaxime, 38% resistance while 61% were sensitive to ceftazidime, 15% resistance and 80 % were sensitive to Meropenem. 10 % resistance to and 86% sensitive to Imipenem, 3.5% resistance and 96% sensitive to tazocin and 6.3 % resistant while 87% were sensitive to sulzon, using disc diffusion method. **Conclusions:** Meropenem, Imipenem, tazocin and sulzon were formed to be the most effective agents isolated *P. aeruginosa* isolates. The results of the bacterial isolates did not showed 100% susceptibility against any of the tested antibiotics. In this study, it is concluded that Tazocin and Sulzon is the choice of drug for the SSI of diabetic patients.

INTRODUCTION

Surgical site infections (SSI) are the most frequently occurring nosocomial infections, the sources of nosocomial infections are the skin tissues but most often spread to soft tissues, urinary tract, respiratory system, and infections related to the bloodstream. Usually, surgical wound infections initiate from the fifth to the tenth day proceeding with surgery. There are numerous reasons beyond post-operative infected wounds including; non-sterilized surgical equipment's, unhygienic surgical operating areas, and inadequate medications. The results can be analyzed through different combinations of symptoms of infection such as erythema, warmth, pain, tenderness, drainage, and, swelling [1]. Mostly post-operative wound infections are hospital-acquired and they are varying between hospitals and even within different wards of a hospital and are largely associated with morbidity and mortality of the patients. Hospital-acquired infections are mostly limited to the surgical site [2] *i.e.* suture line but may extend to the operated body tissues because the infectious microbes are not only variable in nature but also in their pathogenicity [3]. These types of infections are also dependent on the types, surgery

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location, and also medications received for the eradication of micro-organisms [4]. Furthermore, after urinary tract infections the surgical site infections are the second most frequently hospital acquired infections. Literature showed that pathogens involved have not changed during the last decade, amongst the species *Staphylococcus aureus, Staphylococci (CoNS), Enterococcus spp*. Coagulase-negative, and *Escherichia coli* were the most common [5]. Moreover, *S. aureus, Enterococci* and *Staphylococcus* are coagulase-negative *Gram-positive* (G+ve) bacteria that are also found to play a key role in spreading the nosocomial infections [6]. Often the microbes invade the other tissues near wounds because of the closed correlations and similarities between different microorganisms of relevant tissues [7]. In superficial incisions, *S. aureus* is mostly observed, colonized by endogenous bacterial flora or pathogens [8].

METHODS

A total of 200 samples were collected from diabetic ward Hayatabad Medical Complex Peshawar, majority were diabetic foot and screened out for microorganisms passing from different microbiological tests. The identification was done by gram staining and different biochemical tests.

Chemicals and Reagents: MacConkey agar was used for identification of *P. aeruginosa* [9]. For identification of isolates, gram staining was carried out. The reagents of gram staining were safranin, lugol's iodine, acetone alcohol and crystal violet stain. Triple sugar iron agar, citrate agar and oxidase reagent was also used for identification [10]. Nutrient broth and Muller Hinton media were used to refresh cultures and to determine sensitivity pattern by disc diffusion method.

Antibiotics discs were used for disc diffusion method [11]. The discs used in the study included Cefotaxime (CTX) 30 mcg, Ceftazidime (CAZ) 30 mcg, Meropenem (MEM) 10 mcg, Imipenem (IPM) 10 mcg, Piperacillin/Tazobactam (TZP) 110 mcg and Sulzon (SCF) 105 mcg.

Collection of samples: The sample size was 200 and were collected from all the patients with diabetic foot ulcers attending Hayatabad Medical Complex Peshawar. Complete history of the patients was recorded including age, gender and country of origin. The samples were collected by sterile swab sticks from pus of diabetic patients. In moderate to severe wounds, the culture materials were collected either from curettage of debrided ulcer or tissue biopsy [12].

Preparation of Blood agar: In a 100 ml volumetric flask, 5 mg of the media (blood agar) was added. The media was heated so that it gets dissolved and mixed. After mixing it was sterilized at 121° C at 20 PSI for 15 minutes. The media was allowed to cool to 50° C and then 5ml of blood was added. Into sterilized saucers, the media was poured using laminar airflow followed by solidification of the prepared media. To check the sterility, media plates were kept in an incubator for 24 hours at 37°C. The composition of blood agar can be seen in table 1.

Preparation of MacConkey agar: MacConkey agar (5g) was added to 100 ml of pure water in conical flask. Media was heated so that it gets dissolved and mixed. After mixing it was sterilized at 121° C at 20 PSI for 15 minutes. When media was cooled to 50° C, it was poured into sterilized plates inside laminar air flow and allowed to solidify. To check the sterility, media plates were kept in incubator for 24 hours at 37°C. The composition of MacConkey agar can be seen in table 1.

Preparation of Nutrient agar: In a 100 ml of volumetric flask, 2.8 g of the media (nutrient agar) was added. The prepared media was heated so that it gets dissolved and mixed. After mixing it was sterilized at 121° C at 20 psi for 15 minutes. When media was cooled to 50° C, it was poured into sterilized saucers in laminar air flow and allowed to solidify. To check the sterility, media plates were kept in incubator for 24 hours at 37°C. combination of Nutrient agar is given in table 1.

Preparation of 0.5 McFarland's Turbidity Standard: 99.5ml of sulfuric acid (1%) was added to 0.5ml barium chloride (1.175%) to prepare McFarland's Turbidity Standard [13]. It was used for turbidity comparison to the bacterial suspension [14].

Preparation of Muller Hinton Media: 3.8g of media was added to 100 ml of pure water in volumetric flask. Media was heated so that it gets dissolved and mixed. After mixing it was sterilized at 121° C at 20 PSI for 15 minutes. When media was cooled to 50° C, it was poured into sterilized dishes in laminar air flow and allowed to solidify. To check the sterility, media plates were kept in incubator for 24 hours at 37°C. Composition of Muller Hinton Media can be seen in the table 1.

Media	Composition
Blood Agar	Heart muscle infusion (2) pancreatic digest of casein (13) yeast extract (5) sodium chloride (5) agar (15)
MacConkey Agar	Peptone (17) lactose (10) bile salts (1.5) sodium chloride (5) crystal violet (0.001) neutral red (0.03) agar (13.5)
Muller Hinton Agar	Meat infusion (6) starch (1.5) casein hydrolase (17.5), agar (10),
Nutrient Agar (NA)	Yeast extract (3), peptone (15), Nacl (6) agar (12)
Nutrient Broth	Peptone (15) yeast extract (3) sodium chloride (6)
Simmon's Citrate Agar	Sodium citrate (2.0), Ammonium dihydrogen phosphate (ADP) (1.0), Sodium chloride (5.0), Phosphate (1.0) Bromothymol blue (0.008), Di-potassium hydrogen, agar (13.0), Magnesium sulfate (0.2).
Triple Sugar Iron	Peptone meat (5), Meat extract (3), Peptone casein (5) ,Phenol red (0.024), Yeast extract (3), Sodium chloride (5),Thiosulphate (.3) Citrate (.5), Agar (12) , Iron (.5), D-glucose (1), Lactose (10),Sucrose(10).

Table 1: Composition of Media

Inoculation: The samples were inoculated on the MacConkey agar and blood agar in laminar flow hood. Then the plates were placed in the incubator for 24 hrs at temperature 37°C. Positive growth was observed.

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Preservation of bacterial isolates: For further studies the bacterial isolates were preserved and were cultured using nutrient agar slants and kept at 4° C in refrigerator. For further use the isolates were sub cultured.

Isolates identification: For the identification of all the isolates, Gram staining technique and also biochemical tests were adopted. The procedures are given below:

Gram staining: For gram staining, using the sterilized loop, a small drop of sterile distilled water was placed on a sterile glass slide, followed by mixing the bacterial colony water over glass slide. By spreading the bacterial colony a thin smear was prepared. After making a smear it was placed in a safe place to air dry. Heat fixation of the smear was done by passing slide 3-4 times over the flame of Bunsen burner. The fixed smear was covered with crystal violet stain for 1 minute. Stain was washed off with clean water. The coat was enclosed with lugol's iodine for 60 seconds and then cleaned by water. The coat was decolorized with acetone-alcohol for 30 seconds then cleaned
through clean water. For about 1 min the smear was stained with safranin, followed by cleaning with clean water. The slide was dried in air. Simple microscopy was used for identification.

Triple sugar iron (TSI): In 6.75g of media Triple sugar iron was added to 100ml of distilled water in volumetric flask. Media was heated so that it gets dissolved and mixed. After mixing it was autoclaved at 121° C at 20 PSI for 15 minutes. 5-6ml of media was poured into test tubes, the test tubes were plugged and slants were prepared. The initial color of the media was red. Composition of triple sugar iron agar is given in table 1. For sterility testing the slants were incubated at 37° C for 24 hours. The non-contaminated tubes were then inoculated with isolates. Test was considered negative if there was no change in color.

Citrate test: In 2.8g of media was added to 100 ml of the freshly prepared distilled water using the volumetric flask. Media was heated so that it gets dissolved and mixed. After mixing it was autoclaved (at 121° C, at pressure of 20 PSI for 15 min). Followed by pouring 5-6 ml of the media poured into the sterilized test tubes, the test tubes were plugged and the slants were prepared. The initial color of the media was green. Combination of Simon's citrate agar is given in table 1. For sterility testing the slants were incubated at 37° C for 24 hours. The non-contaminated tubes were then inoculated with isolates. No change in color indicates a negative test.

Oxidase test: Oxidase test reagent is readily oxidized so it is freshly prepared when needed. It was prepared by coalescing 0.1g of oxidase reagent to 10ml of distilled water [15]. 2 or 3 drops of oxidase testing reagent were added to a portion of strainer paper trust in a Petri dish. Plantation bacterial segregates were detached using a piece of sticks and smeared on the filter paper. The complete purple color indicates as positive.

Determination of the antimicrobial susceptibility pattern: To determine the antibiotic resistance, agar dilution and disc-diffusion methods were used. Clinical Laboratory and Standard Institute (CLSI) recommendations were followed for such purpose. Nutrient agar broth and Mueller-Hinton agar were the culture media used. During the process of the media preparation, fresh growth of bacteria was suspended in 1 ml nutrient broth and then by using sterilized glass beads, spread on the surface of agar plates. Finally, on the surface of agar plates, the antibiotic discs of different concentrations were placed with equal distances by using sterilized forceps. The data was recorded after incubation at 37°C for 24 hours.

RESULTS

The most common pathogen that can cause nosocomial infections is P. aeruginosa. Cefotaxime, Ceftazidime, Meropenem, Imipenem, Tazocin and Sulzon like broad-spectrum antibiotics were used for the treatment of infection in diabetic patients. Microorganism produces resistance as a natural response to the existence of antimicrobial agents. This resistance in the microorganism is due to the casual use of antibiotics. The current study was made to explore the P.aeruginosa occurrence and susceptibility pattern against the mentioned broad-spectrum antibiotics against the infection of the diabetic patient. The identification results of the different isolates are shown in table 2.

S.No	Media Used	Identification Results
1	Blood Agar	Spreading Colonies, Large Flat
2	MacConkey Agar	Pale Color Colonies
3	Oxidase Test	Deep purple line
4	Triple Sugar Iron (TSI) Test	Red color slant Red butt
5	Citrate Test	No clour change in the media i.e. green color

Table 2: Identification results of Isolates

The specimen was inoculated on Blood agar and MacConkey agar for the test. MacConkey agar and Blood agar representing the P. aeruginosa growth, that appears in the form of colonies with large, spreaded, flat colonies having pale color as shown in figure 1-3

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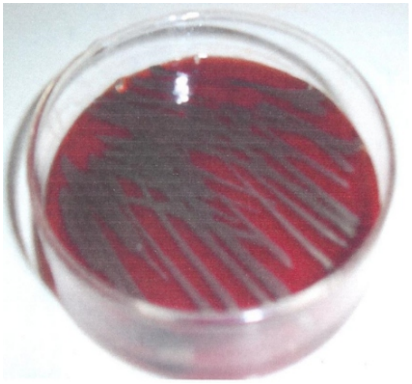

Figure 1: P.aerugionsa colonies on Blood Agar

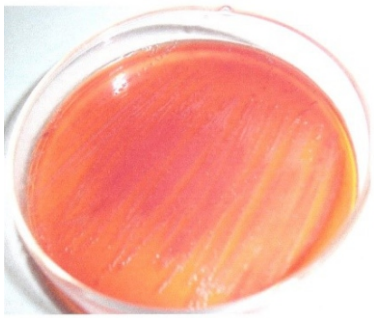

Figure 2: P.aerugionsa colonies on MacConkey Agar

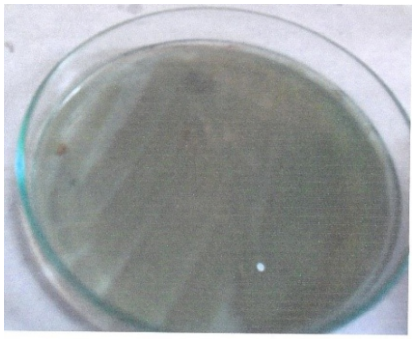

Figure 3: P.aerugionsa colonies on Nutrient Agar

After exposure to the Gram staining and different types of biochemical standards and microscopic examination the results indicated that the isolated microorganism was P. aeruginosa. The biochemical tests including citrate test, oxidase test, and TSI test were performed for the recognition of isolates. The freshly prepared oxidizing reagent was used for oxidase test. The deep purple color was an indicator of positive results. Hence this test confirms the existence of P. aeruginosa. Itwas used for citrate test. In case of positive results the green color of the sample changes to blue. No color changes were observed in case of P. aeruginosa. It was confirmed in negative citrate test. Simmon's citrate agar is the source of citrate presence. Bromothymol indicator changes color from green to blue by citrate-using bacteria. Change in color was not observed, as the P. aeroginosa did not utilize citrates (Figure 4).

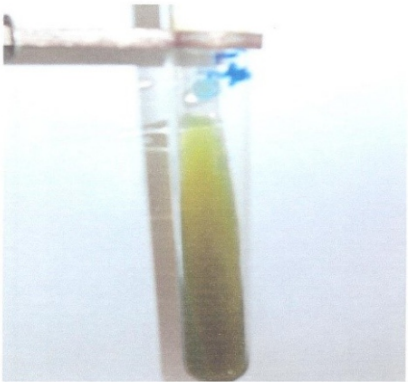

Figure 4: Citrate Medium (Green color)

For isolates identification, triple sugar iron agar test was performed as well [16]. Three sugar (sucrose, lactose and glucose) and iron are the components of this media. An acid is formed by the bacteria as the sugars are fermented which changes the indicator color from red to yellow color. No sugar is fermented by *P. aeruginosa*, hence changes in the media color were not observed. The presence of *P. aeruginosa* was confirmed by negative result. The results of the current data showed that the prevalence of the *P. aeruginosa* was confirmed in 112 patients out of the total 200 patients with a percentage of 56%. Among the studied population male patients were dominant (82 males) as compared to 30 females, w.ith a ratio of 2:1. The rate of diabetic patients infected with *P. aeruginosa* in males was higher due to higher exposure of males to the outside environment as compared to females. Different age group patients were targeted with the age group of 20-60. According to the results of this study, the prevalence of infection rate was higher in patients having between the age group of 36-50 (n=51), followed by patients between the age of 20-35 having (n=22) and patients with age 51 and above had rate of infection 39. The rate of the infection was more in hospitalized patient (96) as compared to OPD patients which was only 16 (i.e. 14%), with a ratio of 4:1 (Table 3). This results in table 3 showed that due to long stay in the hospital the prevalence was more due to exposure to the *P. aeruginosa* having higher infection burden in hospitalized patients with risk factors from bed, unsterilized surgical instruments and other accessories used in hospital which were not properly sterilized.

Patients	Frequency	%
Indoor	96	86
Outdoor	16	14

Table 3: Frequency and percentage of Indoor and Outdoor diabetic patients infected with P. aeruginosa

The samples (mainly from the foot) from the infected diabetic patients were collected followed by isolation of the P. aeruginosa. The disc diffusion method was used to check the susceptibility of isolated P. aeruginosa against

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different antibiotics. After evaluation of the results, the highest percentage of the resistance was found against Cefotaxime (30 mcg) (CTX) followed by Ceftazidime (30 mcg) (CAZ) which was 38%, while the least cases were reported against using Tazocin (110 mcg) (TZP) (Table 4).

Antibiotics	Resistant antibiotics	% of resistant antibiotics
CTX	75	67
CAZ	43	38
MEM	17	15
IPM	11	10
TZP	4	3.5
SCF	7	6.3

Table 4: Susceptibility Pattern of Antibiotics

As shown in table 5 the P. aeruginosa was isolated from 112 diabetic patients, the highest resistance was noted against cefotaxime (CTX) which is about 67%. While against ceftazidime (CAZ) the resistance reported resistance was 38%, the resistance to meropenem (MEM) was 15 % and against sulzon (SCF) that resistance was a bit low as 6.3%. Among 56% of the strains, multidrug resistance was noted against six antibiotics. The results of the current studies depicted that no single antibiotic among the selected ones was 100 % sensitive against P. aeruginosa. The least resistance was recorded against tazocin (TZP) which was noted to be 3.5 %.

Antibiotics	Conc/Disc (mcg)	% Resistance	% Intermediate Resistance	% Sensitivity
Cefotaxime	31	67	1.8	31
Ceftazidime	30	38	01	61
Meropenem	10	15	4.5	80
Imipenem	10	10	4.5	86
Tazocin	110	3.5	0	96.4
Sulzon	105	6.3	7.1	87

Table 5: Antibiotic susceptibility pattern of P. aeruginosa

The disc diffusion method was used to check the susceptibility of P. aeruginosa against cefotaxime and the results showed that 67 % of the isolates were resistance to CTX and 1.8 % had intermediate resistance, while sensitive isolates were 31.3%. Susceptibility of P.aeruginosa strains were checked against the antibiotic (CAZ). From the data it was observed that 1% of the isolates were found intermediate resistant to the CAZ. The rate of sensitivity of the bacterial strain against CAZ was 61% while 38% isolates showed resistance to Ceftazidime. It is reported that 41% isolates were resistant to CAZ.Results showed that against the antibiotic Meropenem the intermediate resistance expressed by P. aeruginosa was 4.5%. Resistant isolates were recorded at 15%, while 80% isolates were sensitive to the antibiotic. Imipinem (IPM) was also used to evaluate the sensitivity pattern of the bacterial isolates. Among the isolates 10 % were found to be resistant while 4.5 % of the isolates were intermediate resistant, while 86% were sensitive to IPM. The sensitivity pattern of our studies shows that 3.5 % of the isolates were found resistant against Tazocin while 96% is sensitive. Sulzon (SCF) was also used to evaluate the sensitivity pattern of the bacterial isolates. Among the isolates, 6.3 % were found resistant while 7.1 % of the isolates showed intermediate resistance.

DISCUSSION

The results of the current data showed that the prevalence of the *P. aeruginosa* was confirmed in 112 (56%) patients out of 200. A study showed total of 77 out of 104 patients in the study. 104 bacteria were isolated from 77 patients. Out of 104 bacteria, 39 (37.5%) were *P. aeruginosa* [17]. The current study showed that among the studied population male patients were dominant. From the results, it is evident that 82 patients were male while 30 were females with a ratio of 2:1. The rate of diabetic patients infected with *P. aeruginosa* in males was higher due to higher exposure of males to the outside environment as compared to females. Scientist also recorded that the ratio from the isolates for male and female patients were 2:1 which was similar to the results of our study [18]. The diabetic patients infected with *P. aeruginosa* were found greater in local people (93) visiting HMC hospital compared to that in afghan refugees (19). Afghan refugees can visit hospital for treatment and take prescription and go back to their country while local people admitted at hospital and take complete treatment until they are cured. Results showed that due to long stay in the hospital the prevalence was more due to exposure to the *P. aeruginosa* having higher infection burden in hospitalized patients with risk factors from bed, unsterilized surgical instruments and other accessories used in hospital which were not properly sterilized. The samples (mainly from the foot) from the infected diabetic patients were collected followed by isolation of the *P. aeruginosa*. The disc diffusion method was used to check the susceptibility of isolated *P. aeruginosa* against different antibiotics. The highest percentage of the resistance was found against Cefotaxime (30 mcg) (CTX) followed by Ceftazidime (30 mcg) (CAZ) which was 38%, while the least percentage of cases were reported against using Tazocin (110 mcg) (TZP). The *P. aeruginosa* was isolated from 112 diabetic patients, the highest resistance was noted against cefotaxime (CTX) which is about 67%. While against ceftazidime (CAZ) the resistance reported resistance was 38%, the resistance to meropenem (MEM) was 15 % and against sulzon (SCF) that resistance was a bit low as 6.3 %. Among 56% of the strains, multidrug resistance was noted against six antibiotics. The results of the current studies depicted that no single antibiotic among the selected ones was 100 % sensitive against P. *aeruginosa.* The least resistance was recorded against tazocin (TZP) which was noted to be 3.5 %. In our

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study, 67 % of the isolates were resistant to CTX and 1.8 % had intermediate resistance, while sensitive isolates were 31.3%. According to studies of Mohammad and Mojtaba showed *P. aeruginosa* strains isolated from diabetic patients were resistant to cefotaxime with a percentage of 62 and 60, respectively, which was similar to the results of the current study [19]. The rate of sensitivity of the bacterial strain against CAZ was 61% while 38% isolates showed resistance to Ceftazidime. It is reported that 41% isolates were resistant to CAZ, while Meenakshi reported that 33% isolates were resistant to CAZ [20]. Results showed that against the antibiotic Meropenem the intermediate resistance expressed by *P. aeruginosa* was 4.5%. Resistant isolates were recorded at 15%, while 80% isolates were sensitive to the antibiotic. According to scientists *P. aeruginiosa* was 100% sensitive to meropenem [21]. The most effective antibiotic agent against bacteria isolated from diabetic foot infection includes imipenem (IPM), meropenem (MEM) and vancomycin (VM). According to studies of Mojtaba resistance isolates were 60%. Imipinem (IPM) was also used to evaluate the sensitivity pattern of the bacterial isolates. Among the isolates 10 % were found to be resistant while 4.5 % of the isolates were intermediate resistant, while 86% were sensitive to IPM. A study by Wahab showed 56% sensitive to imipenem [22] and Javedh study showed 87% sensitive and 14% resistance, while according to Mojtaba *et al.*, 60% of isolated strains were resistant to imipenem[23]. The sensitivity pattern of our studies shows that 3.5 % of the isolates were found resistant against Tazocin while 96% is sensitive. Piperacillin + Tazobactum showed higher sensitivity to Pseudomonas infections[24]. Sulzon (SCF) was also used to evaluate the sensitivity pattern of the bacterial isolates. Among the isolates, 6.3 % were found resistant while 7.1% of the isolates showed intermediate resistance. The rate of the sensitive results showed that the *P. aeruginosa* was 87% sensitive to SCF. Cefoperazone + Sulbactum showed higher sensitivity to *Pseudomonas* infections[25].

CONCLUSIONS

It is concluded that the most common isolate was P. aeruginosa i.e. 112 (56%). Using the disc diffusion approach P. aeruginosa exhibited the susceptibility against Cefotaxime is sensitive 31% and 67% resistant 23 followed by Ceftazadime 61% sensitive and 38% resistant while Meropenem is 80% sensitive and 15% resistant, Imipenem 86% sensitive and 10% resistant, Tazocin (peperacllin/tazobactum) 96% sensitive and 3% resistant and Sulzon 87% sensitive and 6% resistant. The results of the bacterial isolates did not show 100% susceptibility against any of the tested antibiotics. The current results of these comprehensive studies give us insight to the current state of susceptibility pattern of the clinically important specie (P. aeruginosa) to different groups of antibiotics. In this study, it is concluded that Tazocin and Sulzon is the choice of drug for the SSI of diabetic patients.

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