

PREVALENCE AND ANTIBIOTIC RESISTANCE OF PSEUDOMONAS SPECIES ISOLATED FROM EAR SPECIMENS

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Abstract

Background: Ear infections are common medical conditions caused by various microbes including viruses, bacteria and fungi. Empirical antibiotic therapy is the preferred method for treatment of ear infection. Without knowing the etiology and antibiotic susceptibility pattern can increase antibiotic resistance. The aim was to find the prevalence of *Pseudomonas* species and its antibiotic susceptibility in ear specimens.

Methods: A total of 726 patients with discharging ears attending ear, nose, throat (ENT) outpatient clinic from December 2019 to December 2020 were collected in Abbas Institute of Medical Sciences and associated clinical laboratories, Muzaffarabad. *Pseudomonas* species were isolated and characterized by biochemical and microbial techniques. Antibiotic susceptibility was estimated through Kirby -Bauer disc diffusion method.

Results: *Pseudomonas* species were found in 13.3% (97/726) of the total samples tested. The prevalence of *Pseudomonas* was higher in adults (59/97=61%) as compared to children (38/97=39%). Moreover, the prevalence was higher in male (56/97=58%) than female (41/97=42%). Antibiotic sensitivity data revealed that *Pseudomonas* species were more sensitive towards Tazobactam (90.7%) followed by Imipenem (81.4%), Meropenem (76.2%), Amikacin (73.1%), Gentamycin (49.4%) and Tobramycin (37.1%). Sensitivity towards Ceftazidime, Cefepime, and Levofloxacin was found to be 31.9%, 24.7% and 21.6% respectively in isolates.

Conclusion: *Pseudomonas* prevalence in ear infections is substantial in the region and should be considered in the patients having a history of acute and chronic discharge. Antibiotic susceptibility should be considered for such bacteria instead of use of empirical antibiotics to reduce resistance and treatment failure.

Key Words: Pseudomonas, Prevalence, Antibiotic Resistance, Ear Discharge

INTRODUCTION

Outer and middle ear areas are exposed to external environment and there are increased chances of getting infection and injury to these areas [1]. Eustachian tube is a hollow structure in the ear that connects the middle ear to the back of the throat. Due to infections, eustachian tube is often swollen or blocked with the mucous and the fluid cannot drain [2]. Otorrhea is the drainage from ear and may arise from the ear canal or the middle ear [3]. Ear infections are also called otitis, diagnosed by physical examination and laboratory tests of discharge or pus. Symptoms of otitis media are mild to severe pain in the ear, rashes, irritation and pus from ear and in some cases fever [4].

Apparently, it seems like a minor medical condition but can cause serious complications if a person left without treatment or mis-manage [5]. If duration of microbial colonization and severity increase, then it may lead to serious complications. Ear infection is commonly caused by *Pseudomonas* species. Some bacteria such as *E. coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilis influenza* and common aerobes such as *Peptostreptococcus*, *B. fragilis* and *Prevotella melaninogenica* are also responsible for ear infection. *Pseudomonas* species are Gram negative aerobic rod shape bacteria having length ranges from 2-4µm. They are non-spore forming, motile by polar flagellum. They are non-lactose fermenters, catalase positive and oxidase positive bacteria. Infected humans, animals, contaminated water and soil are the main reservoirs of *Pseudomonas* [6]. *Pseudomonas* infection can be community acquired as well as hospital acquired. Pyocyanin produced by *Pseudomonas* species catalyzes the production of hydrogen peroxide and

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superoxide that causes damage of tissue and may lead to inflammation. *Pseudomonas* ability to form biofilms improves its capacity to bind to and survive in medical devices, environmental surfaces, and airways. The pathogen is also involved in blood infection because of transfusion of contaminated blood, blood products and fluids [7]. *Pseudomonas* species are generally considered as an opportunistic pathogen and cause infections in immune compromised patients such as acute leukemia, HIV/AIDS, burns, wound, cystic fibrosis, severe chronic obstructive pulmonary disease, bronchiectasis, organ transplants, diabetes mellitus and intravenous drug users [8]. *Pseudomonas* species can infect different body parts such as ear including otitis externa and media. It can impact respiratory tract in which it causes pneumonia, responsible for infection in bloodstream, also involve heart by causing endocarditis, it can also infect CNS and cause meningitis and brain abscess. It is also responsible for urinary tract infection and skin infection [9]. Antibiotics are the agents which kill or stop the growth of microorganisms and are grouped based on type of microorganism and their antimicrobial characteristics. Antibiotics are the most widely used medications to treat bacterial infections, but they are often misused by physicians, medical professionals, and health care staff due to poor prescription procedures and the treatment of infections other than bacterial infections [10]. Antibiotics often used for treatment of ear infection caused by *Pseudomonas* species include Amikacin, Imipenem, Meropenem, Ceftazidime, Tobramycin, Gentamycin, Levofloxacin, Tazobactam and Cefepime.

Pseudomonas species are intrinsically resistant to several groups of antimicrobials and can also establish resistance and impose challenge during treatment [11]. Due to antimicrobial resistance, the effect of antibiotics has decreased which may lead to difficulty in patient's treatment, duration of illness can be prolonged and increases mortality rate. The prevalence and antibiotic sensitivity pattern of *Pseudomonas* species causing ear infection is different in various geographical areas, climate conditions as well as selection of types of antibiotics [12]. The pathogen has gained resistance against many antibiotics, but growth of *Pseudomonas* species isolates has become a serious problem with Extended Spectrum β -Lactamases (ESBLs). Thus, accurate identification of agent which causes ear infection and antibiotic resistance to such agent must be known [13]. The purpose of this study was to isolate and identify *Pseudomonas* species from patients with ear infection in district Muzaffarabad. Afterwards, the objective was to test the prevalence of ear infection caused by *Pseudomonas* species based on age and gender and to check antibiotic susceptibility of *Pseudomonas* species against commonly used antibiotics to treat ear infection.

METHODS

Sample Collection Procedures

The samples were collected from Abbas Institute of Medical Sciences (AIMS) in district Muzaffarabad and clinical laboratories associated with Abbas Institute of Medical Sciences Muzaffarabad. This study was carried out from December 2019 to December 2020 at AIMS Hospital of Azad Jammu and Kashmir. Questionnaires were used to collect information about patients including age, gender and address. The study subjects of both genders coming to health unit complaining of fever due to ear pain and ear discharge were included as consecutive sampling. All samples were collected by standard microbiological techniques. Ear discharges were collected under strict antiseptic techniques with the help of single use sterile cotton swabs which are commercially available with utmost care to avoid surface contamination (Figure 1).

Sample Processing and Identification

After receiving, these samples were inoculated on MacConkey and Blood agar plates. The plates were incubated overnight at 37°C. Suspected colonies were stored on slant for further tests. After overnight incubation, suspected colonies of *Pseudomonas* species were initially identified based on colonial morphology on blood agar which appeared as large colonies with metallic sheen, mucus like, rough or pigmented and mostly showed beta-hemolysis. On MacConkey agar these colonies do not show lactose fermentation, have colorless but off-white, cream colored flat and smooth colonies. Morphological identification of *Pseudomonas* species was carried out by using Gram staining. In Gram staining, a smear was prepared by using a slide and covers the smear with crystal violet stain. After that smear was washed and flooded with Gram iodine. Then slide was washed again and flooded with decolorizing agent and waited for 20-30 seconds. Slides were washed gently through running tap water and completely drained. The slides were counterstained with safranin and left for about 30-60 seconds. After that slides were washed with tap water and blots were dried and observed under microscope (Figure 2A-2C).

Biochemical Testing

In biochemical testing, different tests were performed to identify *Pseudomonas* species. A clean glass slide was taken and put on it hydrogen peroxide. With the aid of sterile platinum loop, a portion of a pure colony from the bacterial culture was transferred to the slide. The formation of bubbles suggested a positive reaction, while no formation of bubbles indicated a negative result. Bubbles were formed which indicate the presence of *Pseudomonas*. Oxidase test is a key test for identification between the families of

Pseudomonadaceae (Oxidase+) and Enterobacteriaceae (Oxidase-). To perform oxidase test, 2-3 drops of oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) were added to the filter paper. Isolated colonies of suspected *Pseudomonas* were added with the help of a sterile platinum loop and appearance of purple indicated the presence of *Pseudomonas* species (Figure 3A-3C).

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed on Muller Hinton agar (MHA) according to Clinical Laboratory Standard Institute (CLSI) guidelines. The following antibiotics were tested for antibiotic susceptibility i.e. Amikacin, Imipenem, Meropenem, Ceftazidime, Tobramycin, Gentamicin, Levofloxacin, Tazobactam and Cefepime. Antibiotic susceptibility testing was done by following the Kirby-Bauer technique as described by Cheesbrough (1991) using multi-disk (Abteck biological Ltd) and single disks (Oxoid), inoculated plates were tested against different antibiotics. Results were interpreted as recommendation of NCCLS guidelines for disk diffusion which has determined the susceptibility or resistance of the organism to each drug tested. Zone

sizes were measured to the nearest millimeter using a ruler or caliper; include the diameter of the disk in the measurement (NCCLS 2003). First, pure colonies of *Pseudomonas* species were inoculated to sterile nutrient agar broth and were incubated overnight at 37°C. The turbidity of overnight grown culture was adjusted to 0.5% Mcfarland turbidity standard solution. 50µl of adjusted culture was added to Muller Hinton agar plate and was spread uniformly on the entire plate and was kept at room temperature for 20 minutes to dry. Antibiotic discs of known potency were added with the help of sterile forcep. The plates were inverted and incubated for 24 hours at 37°C. The antibiotics were diffused in agar surface and form a clear zone around the disc. The clear zone or zone of inhibition (ZOI) around each antibiotic was measured in millimeter (mm) with the help of a scale. After incubation, metric ruler was used to measure the diameter of the zone of inhibition for each antibiotic used. Then measurement which is obtained from the individual antibiotics was compared with the standard table to determine the sensitivity zone. This measurement determined whether in which gender and age group ear infection is mostly common.



Figure 1: Sterile cotton swab to collect ear pus specimen.

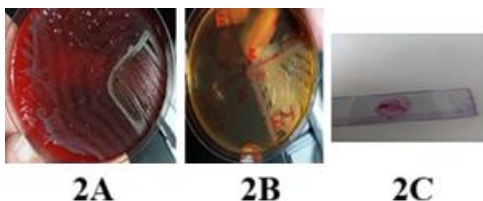


Figure 2: Growth of *Pseudomonas* species on blood agar (2A), Growth of *Pseudomonas* species on macConkey agar (2B) and Gram-stained smear of *Pseudomonas* (2C).



Figure 3: Catalase positive by tube method (3A), Catalase positive on direct plate (3B) and Oxidase positive test (3C).

RESULTS

Biochemical Confirmation

A total of 726 samples were tested for the presence of *Pseudomonas* species. All the samples were streaked on blood and MacConkey agar. Figure 4A-4C showed blood agar with large colonies with metallic sheen, mucus like rough or pigmented and mostly showed beta-hemolysis. On MacConkey agar, *Pseudomonas* do not show lactose fermentation, having colorless but off-white, cream colored flat and smooth colonies.

The colonies were subjected to gram staining. In gram staining, pink color rod shaped bacteria were observed under microscope. These isolated colonies were also tested by catalase by using two methods i.e. direct plate method and tube method as shown in figure 5A and 5B, while oxidase test was performed to confirm the presence of *Pseudomonas* bacteria as shown in 5C. Oxidase and catalase positive bacteria were considered pseudomonas; isolated colonies were further tested for antibiotic sensitivity testing performed on Muller Hinton agar (MHA) (g) for

selection of appropriate dose and type of antibiotic to treat of ear infection caused by *Pseudomonas*.

Prevalence of *Pseudomonas* Species

Out of 726 total tested samples, the prevalence of *Pseudomonas* species was observed in 97/726 (13.3%) samples as shown in figure 6. In addition to month wise prevalence of *Pseudomonas*, we also noted patient age with pseudomonas infection. Out of 97 positive samples of *Pseudomonas*, 59 were adults and 38 were children (1day-15 years). The data showed that *Pseudomonas* were found more in ear samples isolated from adults (above 15 years of age) with 69% prevalence, whereas children were found less prone to *Pseudomonas* ear infection with 31% prevalence. This shows that *Pseudomonas* ear infections are more common in adults as compared to children. Out of 97 positive samples of *Pseudomonas*, 56 were male and 41 were female. The data obtained showed that *Pseudomonas* ear infections are more common in male as compared to female. *Pseudomonas* were found more in ear samples isolated from male with 58% prevalence, whereas females were found less prone *Pseudomonas* ear infection with 42% prevalence. From December 2019 to December 2020, the highest percentage of prevalence of *Pseudomonas* species (26.40%) was reported in the month of December 2020 and minimum percentage of prevalence (4.10%) in the month of June 2020. Prevalence of *Pseudomonas* in ear pus samples by age, gender and month wise are shown in Figure 6.

Resistance in *Pseudomonas* Species

Table 1 represents the sensitivity and resistivity pattern of *Pseudomonas* species. We found that Tazobactam of β -lactamase inhibitor group was highly effective against 88/97 (90.7%) and resistance against 9/97 (9.3%) samples. We also found that Imipenem and Meropenem of Carbapenem group were sensitive in 79 (81.4%) and 74 (76.2%) samples respectively and resistance in 18 (18.6%) and 23 (23.7%) samples. Moreover, aminoglycosides such as Amikacin, Gentamycin and Tobramycin showed sensitivity in 71 (73.1%), 48 (49.4%) and 36 (37.1%) samples respectively and resistance in 26 (26.9%), 49 (50.6%) and 61 (62.9%) samples respectively. Similarly, Cephalosporin group involve antibiotics such as Ceftazidime and Cefepime showed sensitivity in 31 (31.9%) and 24 (24.7%) samples and resistivity in 66 (68.1%) and 73 (75.3%) samples. Levofloxacin belongs to the Fluoroquinolone group of antibiotics showed minimum sensitivity with 21 (21.6%) samples and highest resistivity with 76 samples (78.4%). The data in graphical format is shown in Figure 8.

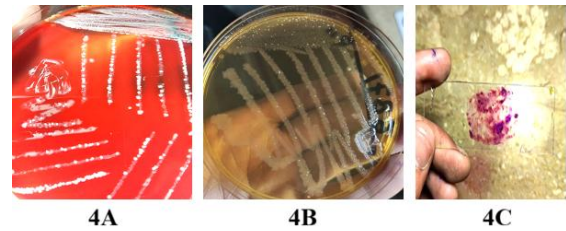


Figure 4: Identification of *Pseudomonas* bacteria on (a) blood and (b) MacConkey agar (c) Gram stain of *Pseudomonas*.

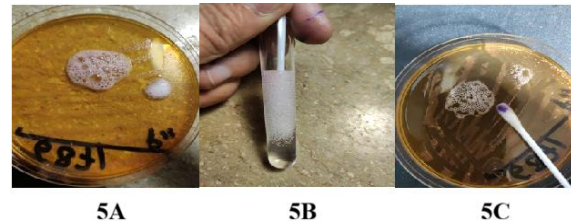


Figure 5: Biochemical tests i.e. (A) and (B) catalase and (C) oxidase test

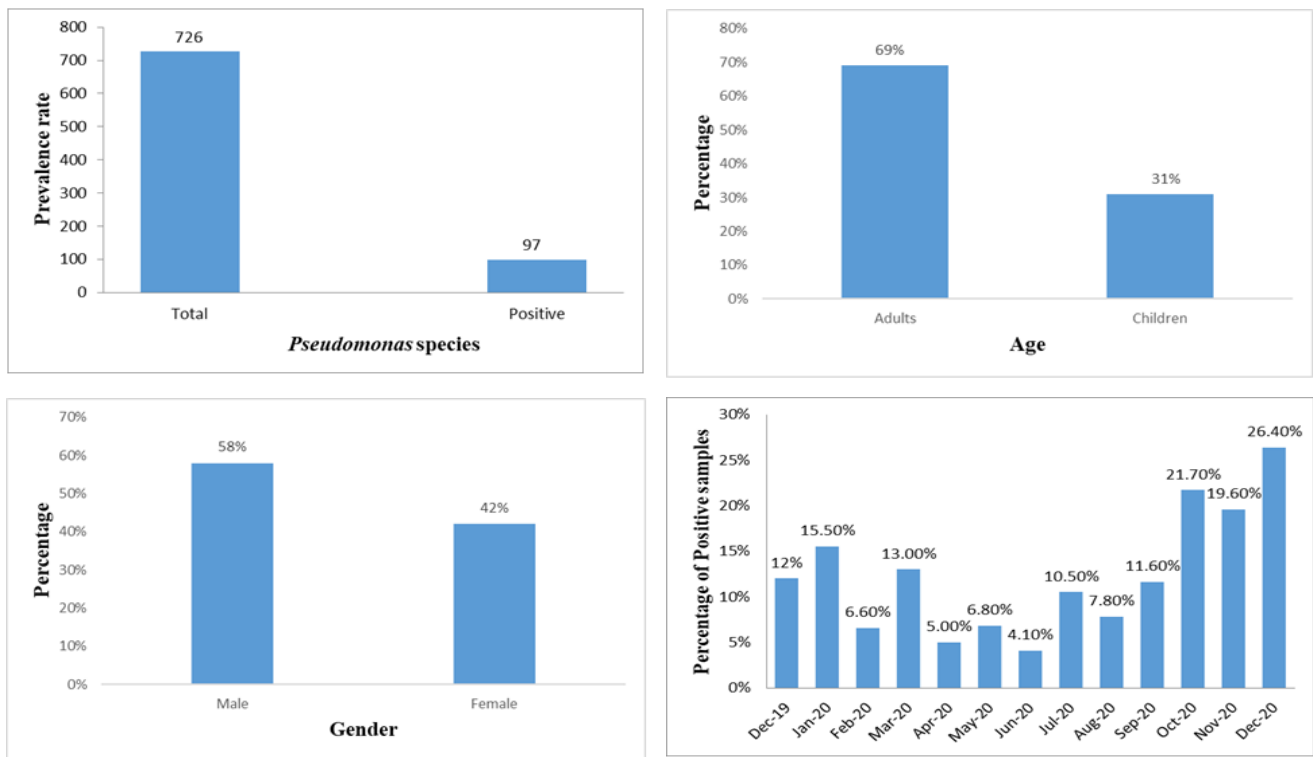


Figure 6: Prevalence of *Pseudomonas* in ear pus samples. Distribution of samples by age, gender and month wise was also recorded.

Table 1: Susceptibility of *Pseudomonas* species against antibiotics

Antibiotics	Sensitivity	Resistance
Tazobactam	90.7%	9.3%
Imipenem	81.4%	18.6%
Meropenem	76.2%	23.8%
Amikacin	73.1%	26.9%
Gentamycin	49.4%	50.6%
Tobramycin	37.1%	62.9%
Ceftazidime	31.9%	68.1%
Cefepime	24.7%	75.3%
Levofloxacin	21.6%	78.4%

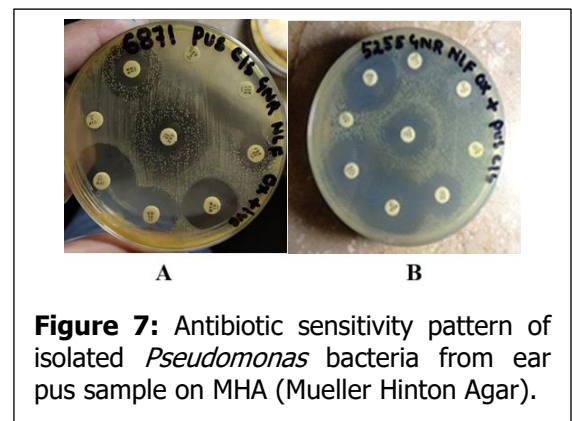


Figure 7: Antibiotic sensitivity pattern of isolated *Pseudomonas* bacteria from ear pus sample on MHA (Mueller Hinton Agar).

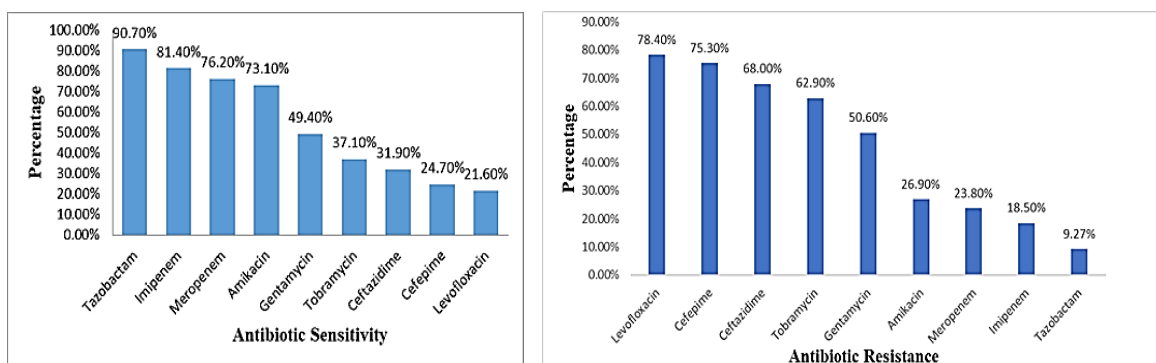


Figure 8: Antibiotic susceptibility and resistance percentage against *Pseudomonas* isolates.

DISCUSSION

Ear infection associated with frequent severe disability, hearing impairment and death is a public health threat in developing countries. Ear discharge sample is one of the most requested specimens for culture and antimicrobial susceptibility tests from clinical settings [14]. *Pseudomonas* are one of the major causes of ear infections. In developing countries like Pakistan, this type of infection is very common, and its eradication is a challenge, as *Pseudomonas* has become resistance to various antibiotics. In this study, the prevalence rate of *Pseudomonas* species from ear specimens was found to be 13.3% from the month of December 2019 to December 2020. The prevalence rate of this study is different with the study conducted in Pakistan at the Microbiology Department of Abasyn University, Peshawar in 2012 where 42% isolates were positive for *Pseudomonas aeruginosa*. The difference of prevalence rate may be due to some environmental factors such as clean water, soil free of pollution of AJK which are different from various regions of Pakistan.

According to this study, gender wise distribution of ear infection caused by *Pseudomonas* in males was substantially higher (58%) than females (42%). Cigarette smokers are at high risk of ear infection as compared to non-smokers. Research by Harner et al found that cigarette smoking greatly affects the auditory apparatus and also causes dysfunction of eustachian tube. As the ratio of cigarette smoking is more in male than female in Azad Jammu and Kashmir, this can be a reason that male is more affected by ear infection as compared to female in this study. Another reason of low ratio of ear infection in female is that they use to cover their ears by hijab in Azad Jammu and Kashmir. So, their ears remain protected by foreign particle as compared to male. This study showed almost similar results compared to another local study in which 61% male and 38% female were infected. According to the study conducted in Bangladesh in 2007, which isolated 54.2% from females and 45.8% from males [15]. This difference in studies may be due to different geographical areas and socioeconomical conditions. In this research, age wise distribution was also studied where lower rate of ear infection was found among patients from 1 day to 15 years of children i.e. 31% as compared to adults (>15 year) i.e. 69%. Similarly, according to a local study where ear infection is in higher proportion in children [16].

The sensitivity of *Pseudomonas* species in this study showed that antibiotic of β -lactamase inhibitor group (tazobactam/ piperacillin) had highest sensitivity (90.7%) to *Pseudomonas* species. These results showed some differences as compared to two studies

conducted in Turkey where tazobactam is resistant [17]. In this study, susceptibility patterns of Cephalosporins such as Ceftazidime and Cefepime were 31.9% and 24.7% respectively. Data is same as the study conducted by Javia et al. [18] where resistance to Cephalosporins were shown. The increase in resistance to Cephalosporins may be due to bacteria involved in the production of extended spectrum β -lactamases. This study disagreed with the findings obtained by study carried out by Gales et al [19] in Egypt, Cephalosporin such as Cefepime was the most sensitive antibiotic against *Pseudomonas* species. In present study, Carbapenems such as Imipenem and Meropenem were sensitive i.e. 81.4% and 76.2% respectively. Studies carried out at the University of Prishtina, Kosovo and Kahraman Maras, Turkey found similar results in which the isolates showed low resistance to Imipenem and Meropenem. However, different results shown by study conducted in India found much increased resistance to Imipenem (66.6%) [20]. The study of Javia et al also showed resistance to these antibiotics [18]. However, a recent study by Rashid et al found resistant strains of *P. aeruginosa* to meropenem [21]. This difference may be due to differences in geographical distribution or may be due to the frequency of exposure of the pathogen towards antibiotics in different regions, further nation wise study is important.

Amikacin (73.1%) showed sensitivity towards *pseudomonas*, whereas Gentamycin (49.4%) Tobramycin (37.1%) were less responsive to *pseudomonas* species, this was different with study in Motayo et al [22], in which *Pseudomonas aeruginosa* showed the highest sensitivity to Gentamicin and were highly resistant towards Amikacin. Another study conducted by Gul et al, where Gentamicin (70%), Amikacin (92%) were most effective [23]. In this study, Fluoroquinolone antibiotics such as Levofloxacin showed highest resistance i.e. 21.6%. In the study findings by Gautam et al where *Pseudomonas* species also showed resistance to Levofloxacin [24].

On comparing the sensitivity patterns of these antimicrobials, it was found that there is a considerable difference in the sensitivity pattern among these studies. This indicates that the sensitivity pattern changes from hospital to hospital and population to population. Thus, as emphasized by various international authorities, every hospital should have its individual antibiotic sensitivity pattern since the standard antibiotic sensitivity pattern may not hold true for every area [25]. This study showed that increased resistance to antibiotics is still a load on developing countries such as Pakistan especially against *Pseudomonas* species which can be community acquired as well as hospital acquired. Thus, it is important to develop the epidemiological

disease control system and antibiotic therapy within the rural areas of developing countries to prevent infectious disease.

To conclude, ear infection remains a public health issue in Muzaffarabad Azad Kashmir. The prevalence is far more than the threshold used by the World Health Organization to qualify as a public health issue. However, many parents were unaware of its presence in affected children. It is suggested to have a strategy to be aware of and guide the public and press regarding this disease to have awareness programs related to ear infection. We also observed that whenever person fell ill, many of them preferred use of traditional medicines. It is necessary to guide public about ear infection and to develop early care seeking from qualified and competent medical staff. This data helped to identify prevalence and characterization of antibiotic susceptibility of *Pseudomonas* species. This not only helped us in overcoming the resistance problem faced in the basic health unit of remote areas but also will be helpful for the clinicians and the practitioners in suggesting the best appropriate antibiotic and dose needed to treat the antibiotic resistance strains.

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Competing Interests: Not Applicable

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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