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# PREVALENCE OF PROTEUS SPECIES AND EVALUTION OF THEIR ANTIMICROBIAL SUSCEPTIBILITY IN VARIOUS CLINICAL SAMPLES OF PATIENTS ATTENDING SREE BALAJI MEDICAL COLLEGE AND HOSPITAL IN CHENNAI

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**ABSTRACT**

In this study we screened the 5077 samples collected from patients who attended SreeBalaji Medical College and Hospital, Chrompet, Chennai from Jan 2014 to Jan 2015 for proteus species. We found that the prevalence of proteus species to be 4.35%. Males were more vulnerable than females in acquiring Proteus infections. Higher prevalence of proteus fell in the age group of 15-40years at 6.33%. Results showed that the greatest number of Proteus Spp isolates from clinical specimens were from pus representing 5.3%. Proteus mirabilis was the most frequent species isolated in the entire specimen 64.6%. Thirteen different antibiotics representing different families of antibiotics were tested on Proteus Spp infections. The proteus specimens were highly sensitive to Meropenem and were highly resistance to Ampicillin. All cefoxitin resistances (85 samples) were taken for Amp C beta lactamase screening, from these 12 samples confirmed to be an Amp C positive by Amp C disk test. Meropenem is superior to other antibiotics for the treatment of infection due to AmpC beta lactamase producing Proteus species.

**Keywords:**proteus species, cefoxitin, ampicillin

## Introduction

Proteus species, belonging to the tribe Proteeae of Enterobacteriacea family, are gram negative bacilli which are catalase positive, oxidase negative, actively motile, non-capsulated, non-spore forming and have the ability to cause disease (1). They are widely distributed in environment and are also part of normal intestinal flora. Since the pathogen has various modes of transmission (2) they are among the most commonly implicated organisms both in nosocomial infections and community acquired infections (3). The genus Proteus has four named species: P. mirabilis, P. vulgaris, P. penneri and P.myxofaciens (4). Proteus is transmitted from various incriminating sources such as soil, contaminated water, food, healthcare personal, hands of patients, equipment and even intravenous solutions (5). Due to their wide habitation they can cause infection in different anatomical sites in the body (4). They are widely seen in urinary tract infections (UTIs), wound infections, bronchoalveolar lavage, epidural ulcers, long term indwelling catheters, body fluids, ear and vaginal swabs, sputum, pus (4).

P. mirabilis accounts for 90% of the Proteus infection in humans, most commonly obtained from urinary and wound infections, it is however not involved in nosocomial infection as do the indole positive species (1). P. penneri infection is rare in humans and confined to infection of urine, wounds in abdomen, groin, neck and ankle (1). P. vulgaris causing UTIs have higher resistance to commonly used antibiotics (6). Proteus species cause significant clinical infections that are difficult to eradicate, especially from people with complicated wounds, who are on long term catheterization, and in immuno-compromised patients as they have the ability to carry the genes encoding antibiotic resistance (1).

AmpC beta-lactamases is an important cephalosporinase, clinically, produced in several Enterobacteriacea conferring resistance to penicillins, cephamycins and beta lactam- beta

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lactamase. Microbes acquire these enzymes by horizontal gene transfer of the plasmid DNA. Persistent treatment with antibiotics leads to the genesis of these enzymes. Several Amp C enzyme producing bacteria are retrieved from hospitalized patients after several days of hospitalization, yet there is ignorance on its clinical consequences and hence remain concealed which are liable for various nosocomial infections in hospitals (6). There is an increase in the infections caused by Amp C and these Amp C producing organisms can act as a hidden reservoir for Extended spectrum Beta lactamases ESBL(7).

As there are not many studies done to identify the prevalence of Proteus, especially in Tamil Nadu, the current study was done to detect the prevalence of Proteus in various clinical samples collected in SreeBalaji Medical College and Hospital, Chennai and to evaluate their antibiotic susceptibility.

## MATERIALS AND METHODS

We processed all the clinical samples, including urine, wound discharge, ear swabs, sputum and blood, collected from patients attending the Sri Balaji Medical College between 01-01-2014 to 31-01-2015 and sent for microbiological analysis, for Proteus species. We also collected the basic demographic data of the patients during sample collection. We inoculated all the samples in strict aseptic conditions on plates of Nutirent agar, blood agar and MacConkey agar and incubated for 37°C for 24 hours. We recorded the morphological characteristics of the colonies such as shape, size, colour, pigmentation and hemolytic nature.

We biochemically tested the suspected Proteus colonies for nitrate reduction, H 2S gas production, methyl red and urease reactions, lactose fermentation. The production of Indole was used to isolate P. vulgaris from other species. We further tested the susceptibility of the Proteus isolates to various microbiological agents such as Ampicillin, Cefiximine, Cefuroxime, Ceftriaxone, Aztreanom, Nitrofurantoin, Nalidic Oxide, Gentamicin and Amikacin by Modified Kirby-Bauer disk diffusion method.

**Methodology:**

**Gram Stain:** Smear were made from all samples expect blood, heat–fixed and stained by gram stain. Smears were examined for the presence of pus cells and Gram–negative organisms.

**Culture:**

Samples were inoculated with standard loop on Nutrient agar (NA), Mac Conkey agar (MAC) and blood agar (BAP). Blood samples were inoculated into Brain - Heart Infusion (BHI) broth and incubate for 24hrs. On the next day, they were sub cultured onto nutrient agar, MacConkey agar and blood agar.

**Examination of subcultures:**

After 24hrs, the plates were examined for the presence of growth. Preliminary identification of organism was made by colony morphology using hand lens. In case of mixed growth, the relative degree of growth of growth of each species was noted. Depending on the morphology of colonies, the presumptive identification of the organism was made.

**Proteus Mirabilis: -**

- On MAC--- Non-mucoid, yellow colonies [Non- Lactose Fermenting colonies]

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- On NA---Moist Translucent colonies, with fishy odour and swarming present
- On BAP---swarming present

**Proteus Vulgaris; -**

- On MAC---Non-mucoid, yellow colonies [Non- Lactose Fermenting colonies]
- On NA---Moist Translucent colonies, with fishy odour and swarming present
- On BAP---swarming present

**Then the growth was subjected to: -**

- Gram stain- to identify gram positive and gram negative organisms
- Hanging drop- To find out motile and non-motile organisms.
- Preliminary test like Oxidase, catalase test was performed.
- Members of the species were identified based on biochemical tests and sugar fermentation tests.

If no growth occurred, the plates were examined after further incubation for another 24hrs before reporting as no growth. Samples showing Proteus species in culture which were confirmed by the biochemical tests were included in the study and were further processed.

**Catalase Test:**

Small amount of the culture to be tested were picked with a clean, sterile glass rod and placed inside the small test tube containing 3% hydrogen peroxide (H2O2) solution. The test was interpreted as positive if immediate, sustained effervescence was observed (8-10).

**Oxidase Test:**

Loop full of the colony to be tested was taken and smeared over the wet filter paper strip containing 1 % tetramethyl-para-phenylenediaminedihydrochloride. ATCC Pseudomonas aeruginosa 29212 and ATCC Escherichia coli 25923 were used as positive and negative controls respectively. The test was interpreted as positive if purple colour developed at the inoculation site within 10 seconds and as negative if the colour did not develop or developed after 10 seconds.

**Table 3**: The isolates belonging to the family Protecea were bio-chemically identified by the following tests

| **S.No** | **Tests** | **Proteus Mirabilis** | **Proteus Vulgaris** |
|---|---|---|---|
| 1 | Indole | Not Produced | Produced |
| 2 | Citrate | Utilised | Utilised |
| 3 | Urease | Hydrolysed | Hydrolysed |

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| | | | |
|---|---|---|---|
| 4 | Voges-Proskauer [VP] | Negative | Negative |
| 5 | Methyl Red[MR] | Positive | Positive |
| 6 | TSI | Alkaline slant/acid butt | Alkaline slant/ acid butt |
| 7 | GAS | Not produced | Not produced |
| 8 | Hydrogen sulphide | Produced | produced |
| 9 | Phenyl pyurate deamination | Deaminated | Deaminated |
| 10 | Xylose | fermented | fermented |

**Storage of Antimicrobial discs:**
The antibiotic storage container was refrigerated at 40-80c or kept frozen at 14c. Beta lactam antibiotics were stored frozen. Disc containers were brought to room temperature before use. Catrige of disc placed in a tight sealed container.

**Preparation of turbidity standard:**
McFarland standards prepared by adding specific volumes of 1% sulphuric acid and 1.175% barium chloride to obtain a barium sulphate solution with a specific optical density. The most commonly used is the MC Farland 0.5 standard, which contains 99.5ml of 1% sulphuric acid and 0.5 ml of 1.175% barium chloride. This solution is dispersed into tubes comparable to those used for inoculums preparation, which are sealed tightly and stored in the dark at Room temp. The McFarland 0.5 standard provides a turbidity approximately 1.5X 108 CFUml (11).

**Preparation of Inoculum:**
In order to prepare the inoculum, about 3 -5 representative colonies were picked up and inoculated in 4-5 ml of peptone water and incubated at 37°C for 2-6 hrs to attain 0.5 McFarland standard which corresponds to 150 million organisms/ml. If it was turbid, then some more quantity of peptone water was added and adjusted to 0.5 McFarland standard by comparing again a card with white background and contrasting black lines.

**Inoculation of MHA plates (44):**
A sterile cotton swab was dipped into the medium. The swab was rotated several times and pressed firmly on the inside wall of the tube. Excess broth from the swab was removed. A dry surfaced MHA plate was taken. Inoculation was done by streaking the swab over the entire sterile agar surface. This procedure was done three times rotating the plate approximately 60°C each time to make sure an even distribution of inoculum.

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**Application of discs to inoculated agar plates:**
The predetermined battery of antimicrobial disc was placed on agar plates and given mild pressure to ensure complete contact with the agar surface. Discs were distributed evenly 24mm from the centre to centre. Plates were inverted and incubated at 37 oC for 16-18hrs (12).

**Reading plates and interpretation of results:**
After 16-18hrs of incubation, plates were examined. The plates which were satisfactorily streaked with proper inoculums showed uniformly circular zones of inhibition and confluent lawn of growth was seen. The diameter of the zones of complete inhibition was measured using sliding calipers which was held on the back of the inverted petri dish, including the diameter of the disc(13).
The petri plate was held a few inches above a black, non-reflecting background and illuminated with reflected light. The area which showed no obvious visible growth with the naked eye was taken as zone margin. The tiny colonies which were detected only with the magnifying lens were ignored. Discrete colonies that grew within a clear zone of inhibition were sub cultured, re-identified and retested. The size of the zones of inhibition were interpreted by referring to the clinical and laboratory standard institute (CLSI) standards and reported as susceptible, intermediate, or resistant to the agents that have been tested.

**Zone size interpretative chart according to CLSI guidelines (14):**

| S.No | Antimicrobial agents | Symbol | Drug conc(µg) | Zone size in mm | | |
|---|---|---|---|---|---|---|
| | | | | Resistant | Intermediate | Sensitive |
| 1 | Gentamicin | GM | 10 | <12 | 13-14 | >15 |
| 2 | Amikacin | AK | 30 | <14 | 15-16 | >17 |
| 3 | Nitrofurantoin | FU | 300 | <14 | 15-16 | >17 |
| 4 | Nalidic acid | NA | 30 | <13 | 14-18 | >19 |
| 5 | Ofloxacin | OF | 5 | <12 | 13-15 | >16 |
| 6 | cefuroxime | CF | 30 | <15 | 15-17 | >18 |
| 7 | Ceftazidime | CZ | 30 | <14 | 15-17 | >18 |
| 8 | Ceftriaxone | FR | 30 | <13 | 14-20 | >21 |
| 9 | Aztreonem | AT | 30 | <15 | 16-21 | >22 |
| 10 | Cefoxitin | CX | 30 | <14 | 15-17 | >18 |
| 11 | Meropenem | MRP | 10 | <19 | 20-22 | >23 |
| 12 | Ampicillin | AMP | 10 | <13 | 14-16 | >17 |
| 13 | Ciprofloxacin | CI | 5 | <15 | 16-19 | >20 |

**Screening for Amp C beta-lactamase:**
Isolates were screened for cefoxitinsusceptility by the standard disk diffusion method using 30µg disks. Isolates that yielded a zone diameter less than 18mm were suspected to be AmpC producers, described as screen positive, and further subjected confirmatory testing (15).
**Amp C disc test:**
The test is based on the use of Tris-EDTA to permeabilize bacterial cell and release β-lactamases into the external environment. Amp C discs (i.e., filter paper disks containing Tris-EDTA) were prepared in house by applying 20µg of 1:1 mixture of saline and 100 X Tris-EDTA to sterile filter paper discs allowing the discs to dry and storing them at 2 -8oC. The surface of MHA plate was inoculated with a lawn of cefoxitin- susceptibleE.Coli ATCC 25922 according to the

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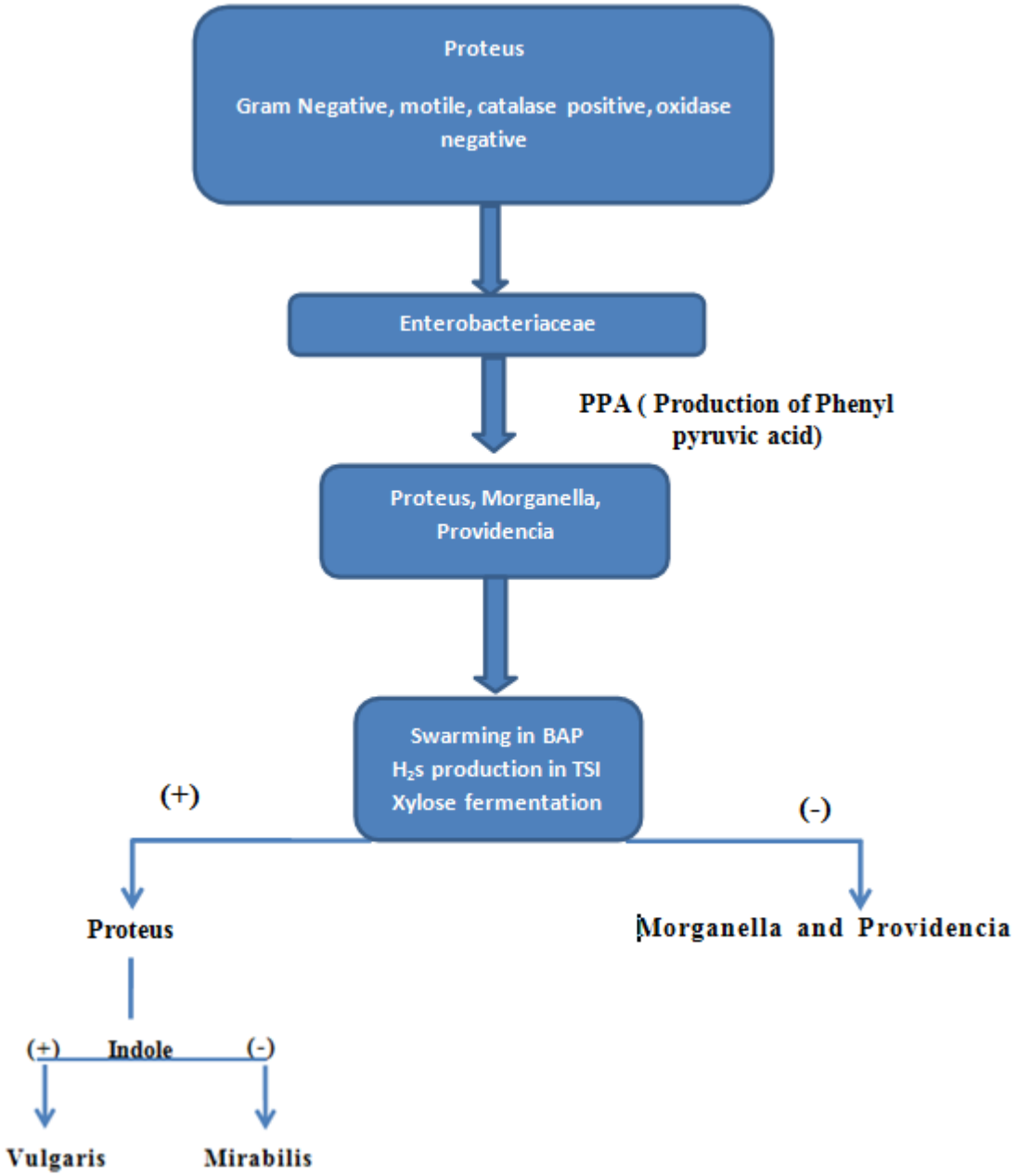
standard disc diffusion method. Immediately prior to use, Amp C discs were rehydrated with 20µl of saline and several colonies of each test organism were applied to a disc.
A 30µl cefoxitin disc was placed on the inoculated surface of the MHA. The inoculated Amp C disc was there placed almost touching the antibiotic disc with the inoculated disc face in contact with the Agar surface. The plate was then inverted and incubation, plates were examined for either a distortion, indicating no significant in activation of cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result).

**Figure 1:**
Flowchart showing identification of Proteus species



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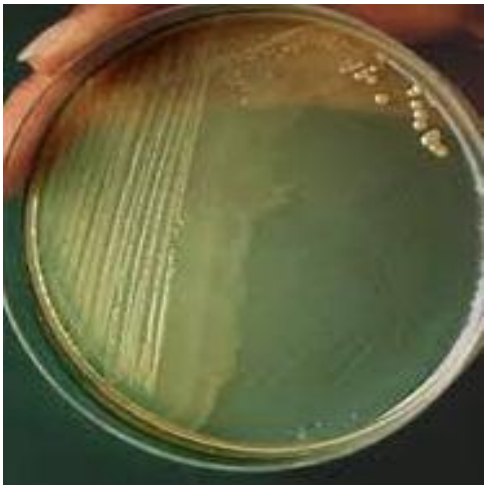

**Figure 2:** Proteus mirabilis showing swarming on Nutrient agar

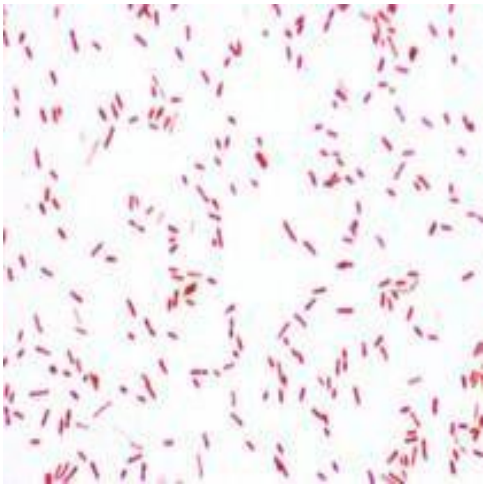

**Figure 3:** Proteus vulgaris showing pleomorphism in gram stain

**Figure 4:** Biochemical reactions of Proteus mirabilis

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Indole- not produced; Citrate- utilized; urease; hydrolysed; TSI- alkaline slant/acid butt; H2S-produced; MMM-motile, not fermented; MR-positive; VP-negative; PPA-positive.

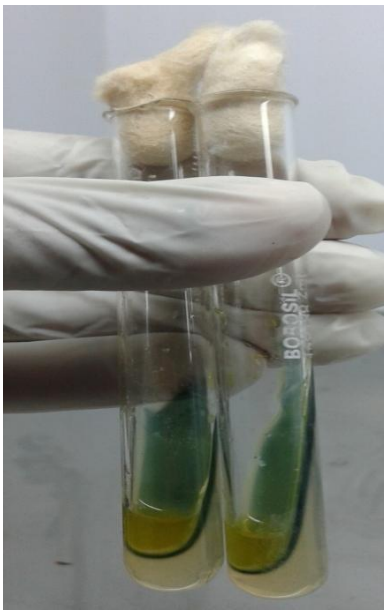

**Figure 5:** Phenyl pyruvic acid Test showing positive for proteus species

**Figure6:** Sugar fermentation reactions

Glucose- fermented; Lactose-not fermented; Mannose- not fermented; Maltose-not fermented; Xylose- fermented

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**Figure 7:** Antibiogram of Proteus mirabilis showing resistance to all drugs

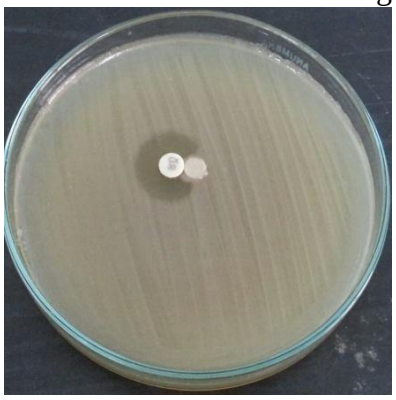

**Figure 8:** Amp C disk test

## RESULTS

We collected 5077 samples from various sites including urine, pus, sputum, blood, body fluids and ear swab. Nearly half of those samples obtained were from the age group of 15 to 40years (Table 1). A majority of the samples belonged to males, 62% (table 1). Higher proportion of the samples consisted of urine (47%) followed by pus (23%) while the ear swab was the least with 2% (Table 1).

**Table 1:** Showing general characteristics of the study samples according to age and sex distribution

| **General characteristics** | **Variables** | **Number #** | **Percentage %** |
|---|---|---|---|
| **Age** | <15 years | 886 | 17.66 |
| | 15-40 years | 2453 | 48.90 |
| | ≥40 years | 1677 | 33.43 |
| | | | |
| **Sex** | Male | 3103 | 61.86 |

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| | | | |
|---|---|---|---|
| **Type of sample** | Urine | 2374 | 47.33 |
| | Pus | 1194 | 23.80 |
| | Sputum | 497 | 9.91 |
| | Ear swab | 104 | 2.07 |
| | Blood | 576 | 11.48 |
| | Body fluids | 271 | 5.40 |

A total of 61samples were found to be contaminated and hence was not included in the study. Thus we processed the remaining 5016 samples for Proteus species and found 209 samples to be positive for Proteus with an overall prevalence of 4.12% (Table 2).

**Table 2:** Prevalence of Proteus among various samples obtained from patients in Jan 2014 to Jan 2015

| **Type of sample** | **No.of samples** | **No.of Proteus Isolated** | **Percentage** |
|---|---|---|---|
| Urine* | 2385 | 115 | 4.82 |
| Pus* | 1207 | 68 | 5.63 |
| Ear swab | 104 | 4 | 3.85 |
| Blood | 576 | 0 | 0.00 |
| Sputum* | 506 | 22 | 4.35 |
| Body fluids* | 299 | 0 | 0.00 |
| Total | 5077 | 209 | 4.12 |

*- specimens inclusive of the 61 contaminated samples

The prevalence of Proteus was high among males with 4.9% while it was 3.1% among the females (Table 3).

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**Table 3:** Prevalence of Proteus among males and females

| Gender | Proteus positive ($n_1$=209) | Proteus Negative ($n_2$=4807) | Proteus Prevalence |
|---|---|---|---|
| **Male** | 151 | 2952 | 4.87 |
| **Female** | 58 | 1855 | 3.13 |
| **Total** | 209 | 4807 | 4.35 |

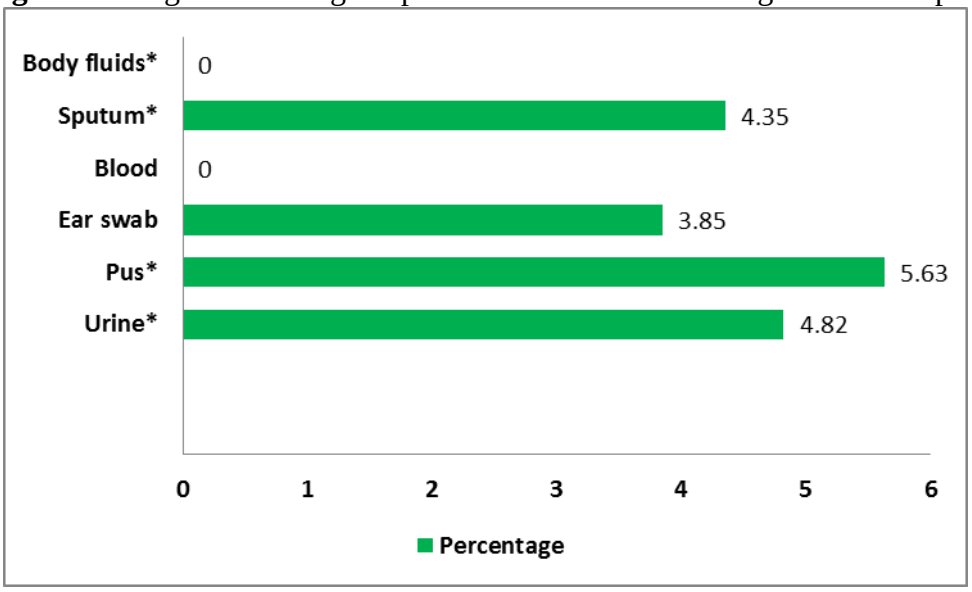
Higher prevalence of Proteus fell in the age group of 15-40 years at 6.33% while it was 2.35% among the age group <15years and 2.63% among the age group ≥ 40years (Table 4).

**Table 4:** Age wise prevalence of Proteus

| Age distribution | Proteus positive ($n_1$=209) | Proteus Negative ($n_2$=4807) | Prevalence % |
|---|---|---|---|
| **<15yrs** | 20 | 866 | 2.31 |
| **15-40yrs** | 146 | 2307 | 6.33 |
| **≥40yrs** | 43 | 1634 | 2.63 |
| **Total** | 209 | 4807 | 4.35 |

The pus samples showed a higher prevalence of Proteus, followed by urine, sputum and ear swab, as shown in Fig 2.

**Fig 2:** Bar diagram showing the prevalence of Proteus among various samples




The prevalence of Proteus mirabilis was higher with 65.5% while 34.5% of the positive samples were of Proteus vulgaris. The prevalence Proteus mirabilis was uniformly high among the various age groups (Fig 3), with the age group <15 years showing nearly double the prevalence of P. mirablis when compared to P. vulgaris. Proteus mirabilis was the most frequent species isolated in all the specimen with the exception of catheter, from which was only isolated Proteus Vulgaris. Among the proteus positive specimens, 72.5% belonged to males while 18% and 9.5% of it belonged to females and children respectively.

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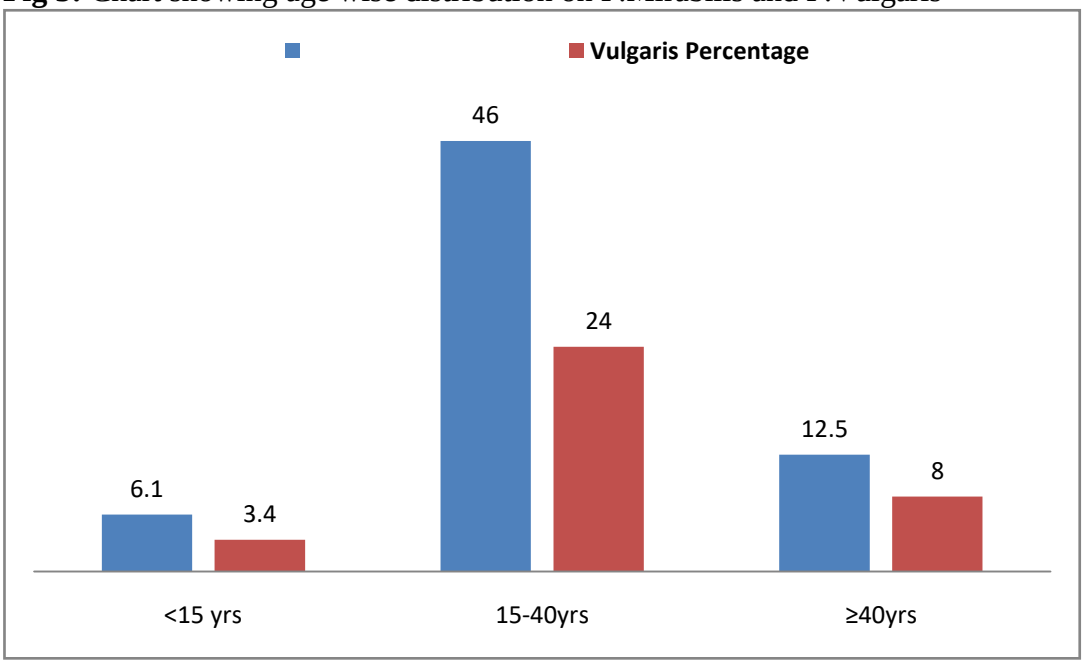
**Fig 3:** Chart showing age wise distribution on P.Mirabilis and P.Vulgaris




The antimicrobial sensitivity tests were also done for all the proteus positive specimens and it was seen they were highly sensitive to Meropenem(73.2%) , ofloxacin(63.2%) followed by ceftriaxone (65.6%) . They were highly resistant to Amipicillin(60.3%), ciprofloxacin (51.7%) and cefuroxime(43.1%). The resistant pattern of Proteus species to 3rd generation cephalosporins were cefuroxime(43.1%),ceftriaxone(25.8%),cefotaxime(21.5%),ceftazidime (16.7%).

**Table 4:** Table showing the sensitivity pattern of various samples

| **Antibiotic** | **Sensitivity** | **Urine** | **Pus** | **Sputum** | **Ear Swab** | **Percentage** |
|---|---|---|---|---|---|---|
| Ciprofloxacin | S | 20 | 38 | 5 | 1 | 30.6 |
| | I | 15 | 20 | 2 | 0 | 17.7 |
| | R | 80 | 10 | 15 | 3 | 51.7 |
| Ofloxacin | S | 81 | 35 | 14 | 2 | 63.2 |
| | I | 22 | 22 | 4 | 1 | 23.4 |
| | R | 12 | 11 | 4 | 1 | 13.4 |
| Gentamycin | S | 49 | 50 | 16 | 2 | 56.0 |
| | I | 14 | 4 | 3 | 0 | 10.0 |
| | R | 52 | 14 | 3 | 2 | 34.0 |
| Ampicillin | S | 29 | 26 | 9 | 1 | 31.1 |
| | I | 10 | 8 | 0 | 0 | 8.6 |
| | R | 76 | 34 | 13 | 3 | 60.3 |
| ceftazidime | S | 80 | 39 | 15 | 3 | 65.6 |

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| | | | | | | |
|---|---|---|---|---|---|---|
| | I | 15 | 19 | 2 | 1 | 17.7 |
| | R | 20 | 10 | 5 | 0 | 16.7 |
| cefotaxmine | S | 78 | 47 | 8 | 2 | 64.6 |
| | I | 16 | 7 | 5 | 1 | 13.9 |
| | R | 21 | 14 | 9 | 1 | 21.5 |
| Ceftriaxone | S | 78 | 29 | 7 | 3 | 56.0 |
| | I | 18 | 12 | 8 | 0 | 18.2 |
| | R | 19 | 27 | 7 | 1 | 25.8 |
| Nalidic acid | S | 51 | | | | |
| | I | 11 | | | | |
| | R | 53 | | | | |
| Nitrofuratoin | S | IR | | | | |
| | I | IR | | | | |
| | R | IR | | | | |
| Cefuroxime | S | 47 | 25 | 9 | 1 | 39.2 |
| | I | 18 | 14 | 3 | 2 | 17.7 |
| | R | 50 | 29 | 10 | 1 | 43.1 |
| Amikacin | S | 43 | 45 | 13 | 2 | 49.3 |
| | I | 15 | 3 | 3 | 1 | 10.5 |
| | R | 57 | 20 | 6 | 1 | 40.2 |
| Aztreonam | S | 49 | 27 | 10 | 0 | 41.1 |
| | I | 17 | 16 | 7 | 2 | 20.1 |
| | R | 49 | 5 | 5 | 2 | 29.2 |
| cefoxitin | S | 47 | 29 | 10 | 1 | 41.6 |
| | I | 19 | 15 | 2 | 1 | 17.7 |
| | R | 49 | 24 | 10 | 2 | 40.7 |
| Meropenem | S | 86 | 49 | 16 | 2 | 73.2 |
| | I | 15 | 10 | 2 | 2 | 13.9 |
| | R | 14 | 9 | 4 | 0 | 12.9 |

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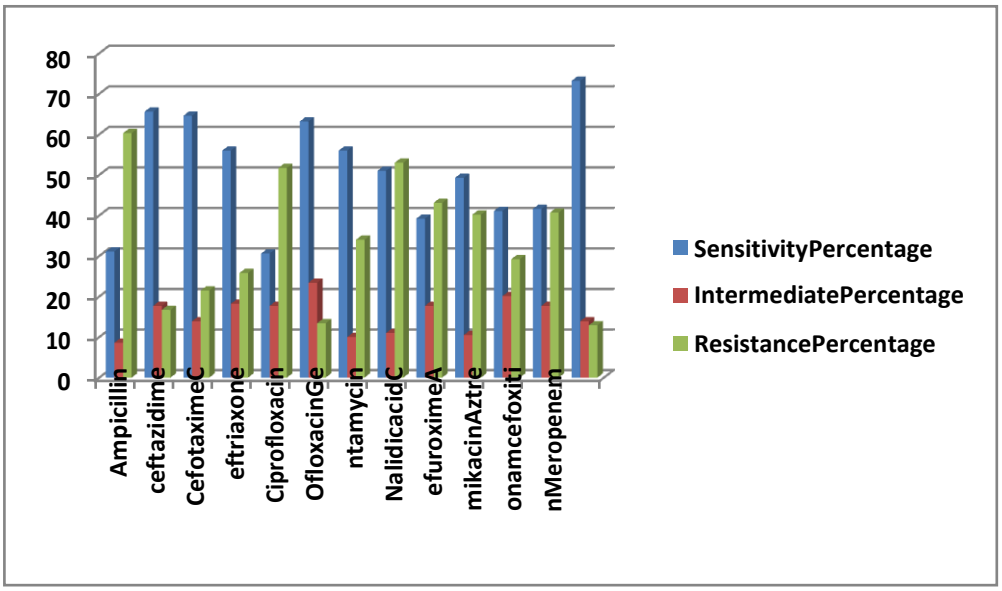
**Fig 3:** Bar diagram showing the antimicrobial sensitivity pattern of Proteus specimens to different antibiotics




85 samples were screening positive for Amp C producers. Among these 85 samples 12 found to be AmpC positive. All the AmpC beta lactamase producers showed 100% susceptibility to Meropenem both in Proteus mirabilis and Proteus vulgaris. All the AmpC producers were resistances to cephalosporins.

## DISCUSSION

The Proteus species has greater clinical importance for its association with higher frequency of community acquired and hospital acquired infections. The increasing resistance to various groups of antibiotics for Proteus species poses a greater challenge in managing the infections. Hence there is a need for continuous survey but there are very few documented information and limited studies available. In this study all age groups were affected with proteus species. More common among 15 -40 years, with the prevalence rate of 6.33%, when compared with > 40 years which is 2.63% followed by <15 years, showing a prevalence rate of 2.31%. But this is in contrast JitendrakumarPandey et al study was the elderly (≥60 years) had higher prevalence rate of 23.21% compared to other age groups (3).

In our study the overall prevalence of Proteus is 4.12 which is a little higher than the prevalence done in recent studies in Saudi Arabia (16) and much higher than a study in Mumbai, India (3). The prevalence of Proteus infection is found to be more among males when compared to females which was similar to the results of the study done by Jitendra et al in 2013 (3). The study also shows the prevalence to be higher among pus samples followed by urine samples which were similar to findings in the study done by Bahashwan et al (16). This study showed P. mirabilis at a higher prevalence than P.vulgaris which coincided with the study findings of Mordi et al done in 2009 (1).

The antimicrobial resistance done among those samples which showed positive for Proteus showed high antimicrobial resistance against Ampicillin, followed by Ciprofloxacin and 3rd

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generation cephalosporin. Many studies showed similar findings including the study done by Bahashwan et al (16).
The susceptibility of all Amp C beta l actamase producing isolates were found to be high for Meropenem of the carbapenaems group, similar to those shown in various studies (17-18).

## CONCLUSION

This study shows the burden of Proteus and its susceptibility pattern to various antimicrobials. Although the prevalence is lower, the fact that Proteus are a major cause of community acquired and nosocomial infections makes it a valuable area of study. The rampant use of antimicrobials and improper dosing has great impact on the antimicrobials in use. Further the dearth in formulating new molecules to fight against these microorganisms is making it all the more important to cautiously use the available antimicrobials in a proper way especially against the largely ignored species like Proteus. Further studies should be done to isolate the other species of Proteus and also to do the antimicrobial susceptibility testing for all of them. Identification of Amp C may aid in hospital infection control and help the physician to prescribe the most appropriate antibiotic, thus decreasing the selective pressure, which generates antibiotic resistance. Meropenem were exceedingly effectual against AmpC producers.
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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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