ISOLATION AND CHARACTERIZATION OF MULTI-DRUG RESISTANCE PROTEUS VULGARIS FROM CLINICAL SAMPLES OF UTI INFECTED PATIENTS FROM MIDNAPORE, WEST BENGAL

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ABSTRACT

Recent global concern is that the inappropriate treatment and misuse of antibiotics leads to the emergence of antibacterial multidrug resistance in Proteus vulgaris and others common pathogenic bacteria. The present study investigates the prevalence of antibiotic resistance among Proteus vulgaris bacterial isolates from urine samples of UTI infected hospitalized patients. Thirty six (36) strains of Proteus vulgaris were collected from twelve hundred sixty (1260) Urinary Tract Infected (UTI) urine samples of the patients admitted to the nearby District Hospital. Characterization of isolated strains was determined by biochemical tests. Sensitivity pattern of isolates were determined by some standard antibiotics. From this study, it was revealed that 59.53% isolates were gram’s negative. Indole positivity was observed for 94.44% of Gram’s negative strains. Isolated strains were 100% positive for methyl red, urease, Phenylalanine deaminase, nitrate reduction, motility, Acid production on D-Glucose, D-sorbitol, Rafinose. 94.44% positivity was seen in urease and gilatinase test. \( \text{H}_2\text{S} \) production seen in 97.22% on Triple Sugar Iron (TSI) agar test respectively. 100% negativity was seen for Voges-Proskauae, Oxidase, Lactose (MacConkey agar), Citrate and Glycerol (Acid production) test. It was also observed that isolates were non lactose fermented on differential media such as MacConkey and CLED agar. Antibiotic susceptibility of characterized P. vulgaris strains were 100% multi drug resistant.

Key-words: Proteus vulgaris, disc agar diffusion, antibiotic susceptibility, multi drug resistant.

1. INTRODUCTION

The human intestinal tract consists of a large variety of aerobic and anaerobic organisms. Majority of the organisms are commensals but there are some obvious exceptions. Outside the intestine, they have a role to play as pathogens which are the most frequent reasons of wound infections, urinary tract infections, nosocomial and various other types of infection. According to American centers for disease control and prevention report about 30% of the all nosocomial pathogens belongs to the enterobacteriaceae family and about 50% of UTI pathogens belong to this group (Jamil et al. 2007). It was a trend in last few years that increasing number organism becoming resistant to several antibiotics among enterobacteriaceae (Cohen-Nahum K. et al. 2010). Proteus species are widely distributed in...
Proteus vulgaris is a pathogen and it was firstly described by Margit Luise Hauser in 1885. Hickman et al. found that three subgroups of proteus species, one group of them was indole negative, salicin negative, esculin negative and had resistant against chloramphenicol disks, designated as Proteus vulgaris (Hickman FW et al. 1981).

In previous study by several researchers found that a variety of organisms will colonize the urine catheters of patients undergoing long term catheterization (Macleod SM et al. 2007) and many of them like Proteus vulgaris are capable of producing urease which generate ammonia from urea and elevate the pH above 8.3 of urine (Stickler D et al. 1998). It causes crystals of calcium and magnesium phosphates to form in the urine and the biofilm that develops on the catheter produce catheter-blocking crystalline biofilms within 40 hours (Broomfield RJ et al. 2009). Earlier DebMandal et al. found that most common antibiotics used for treat of the patients against Proteus vulgaris were resistant in Kolkata region, West Bengal, India. Abuse and improper prescribing policy of antibiotics causes remarkable increase of antibiotic resistance pattern among the P. vulgaris isolates. These types of resistance associated with genetic mutation and intra or inter species transfer of resistance gene through plasmid (DebMandal et al. 2011). Fuchs et al., reported that Proteus vulgaris isolates were susceptible to four board spectrum cephalosporins (Fuchs S. et al. 1996). Frequency of nosocomial infection and UTI cases caused by multidrug resistance P. vulgaris required strong concern of medical practioners and healthcare agencies. Therefore regional studies on pattern of antibiotic sensitivity are very much necessary to overcome this problem. The high prevalence of antibiotic-resistant bacteria harboring diverse resistance traits could represent a potential health risk. The study of antibiotic resistance helps predict future emergence and guide the development of strategies to counteract this resistance. Therefore periodic and comprehensive survey of antibiotic resistance in the environmental bacteria is required. Considering this background the present study was conducted to identify the multidrug resistant Proteus vulgaris and also investigate the drug resistance pattern among those P. vulgaris strains collected from nearby hospital. This study will not only aware medical professionals of this hospital but also aware a major medical authorities to formulate antibiotic prescription policies.

2. MATERIALS AND METHODS

2.1 Culture media and Chemicals
Nutrient agar, Luria broth, Tryptic soy broth, bacterial agar powder, Beef extract, pancreatic digest of casein, blood agar base, Mueller-Hinton broth, MacConkey agar w/o crystal violet, Cystine Lactose-Electrolyte-Deficient (CLED) agar, Simmon’s Citrate agar, N, N, N’, N’-tetramethyl-p-phenylenediamine dihydrochloride Lugol’s iodine, Safranine, Crystal violet and antibiotic discs such as Ampicillin, Penicillin-G, Cephotaxime, Gentamycin, Streptomycin, Tetracycline, Erythromycine, Chloramphenicol, Kanamycine, Oxacillin, Norfloxicin, Niprofloxicin, Amikacin and Imimen were purchased from Himedia, India. Sodium Chloride (NaCl), Hydrogen Peroxide (H2O2), Sucrose, Lactose, Glucose, Galactose, Potassium dihydrogen phosphate (KH2PO4), Di potassium hydrogen phosphate (K2HPO4), Sodium hydroxide (NaOH), Potassium chloride (KCl), were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All the chemicals were purchased from...
Himedia Laboratories Pvt. Ltd., SRL Pvt., Ltd and Merck India Ltd.

2.2 Sample Collection and Transport
One thousand two hundred sixty (1260) UTI patents’ urine samples were collected from patients admitted to Midnapore Medical College and Hospital, West Bengal, India. Urine samples were collected according to patient’s infection history and treatment schedule during the period of two years (1st January 2011 to 31st December 2013). Samples were transported to the laboratory and inoculated in sterile Luria broth (LB) media immediately (Dash et al. 2012).

2.3 Culture of microorganisms
Urine samples with LB incubated in a shaking incubator at 37°C for overnight. After incubation the bacterial cultures were inoculated on Nutrient agar (NA) media plate. Finally single colony isolation was done using differential media like CLED media to differentiate bacterial species (Dash et al. 2012). Isolates were sub cultured on fresh NA plates for further characterization and antimicrobial sensitivity studies.

2.4 Quality control strains
*Staphylococcus aureus* ATCC 29213, *Shigella flexneri* ATCC 12022 were obtained from Department of Microbiology, Midnapore Medical College and Hospital, *Staphylococcus epidermidis* ACTC 5866, *E. coli* ATCC 23509, *E. coli* ATCC 25922, *E. faecalis* ATCC 51299, *E. faecalis* ATCC 29212, *Pseudomonas aeruginosa* 27853, *Proteus vulgaris* ATCC 29906, *Acinetobacter calcoaceticus* ATCC 19606 were obtained from Department of Microbiology, Calcutta Medical college and Hospital. All the reference strains were kept and stored in agar slants at 4°C.

2.5 Screening by Gram’s staining
Gram’s staining was performed according to Duguid, et al. method (Duguid, et al. 1996). At first, on a glass slide bacterial smear was prepared on a glass slide from broth culture media. Then it was heat fixed by slight heating. The slide smear was covered with 0.5% crystal violet and left for 30-60sec. Then it was poured with 1% Lugol’s iodine for 30 sec. Then destaining was done with 95 - 100% ethanol. After that the slide was rinsed with water and 0.1% safranin was poured on it for 2 min. Then the excess stain was washed with water and blotted for drying. Then it was observed under phase contrast microscope (Nikon Eclipse LV100POL). *Proteus vulgaris* ATCC 29906 was taken as positive control for this test.

2.6. Screening agar media
All the bacterial isolates were inoculated on MacConkey agar w/o Crystal violet (Gelatin peptone 1.7 gm%, Bile salts 0.5 gm%, Peptone mixture 0.3 gm%, Lactose 1.0 gm%, NaCl 0.5 gm%, Neutral red 0.003 gm% and Bacteriological agar 1.2 gm%) and incubated at 37°C for overnight. Lactose fomenter bacteria forms Pink-Red colour colony. CLED agar was also prepared for screening test of isolated bacteria depending on the ability to ferment Lactose and Sucrose. *E. coli* ATCC 25922 and *Proteus vulgaris* ATCC 29906 were taken as positive and negative control strains (Eaton et al. 1995).

2.7 Biochemical identification tests
2.7.1 Catalase test
For tube catalase test, 200µl of 3-6% hydrogen peroxide (H₂O₂) was taken in a test tube. A colony of isolates was taken from NA plate with disposable loop and rubbed onto the inside wall of the test tube and examined the vigorous bubbling within 10 seconds. For slide catalase test, 2-3 colonies of isolates was taken from NA plate with sterile loop and spotted onto the centre of a glass slide. One drop of 3-6% H₂O₂ was added on it and observed the vigorous bubbling within 10 seconds. *E. faecalis* ATCC 29212 and *Proteus vulgaris* ATCC 29906 were taken as positive and negative control respectively (MacFaddin JF. 2000).

2.7.2 Oxidase test
Oxidase test of isolates was performed by filter paper method according to Snell et al. (Snell et al. 1999). Briefly, a piece of filter paper was soaked in 1% N, N, N’, N’-tetramethyl-p-phenylenediamine dihydrochloride solution. With a disposable loop some fresh growth of isolates from NA plate was scraped and rubbed onto the filter paper and examined for the blue colour within 10 seconds. *S. aureus* ATCC 25923 and *Proteus vulgaris* ATCC 29906 were taken as positive and negative control respectively.
2.7.3 Indole test
Indole test was done according to standard method (Fingergold et al. 1974). Shortly, bacterial isolates were grown on TSB (tryptic soy broth) for 48 hours. After that Kovac’s reagent was added to those culture media. Development of a red/pink layer on top of the media was considered as positive result; whereas absence of red colour was the indication of negative result. *Proteus vulgaris ATCC 29906* and *Pseudomonas aeruginosa 27853* were taken as positive and negative control strains respectively.

2.7.4 Phenylalanine Agar (PPA) test
Phenylalanine Agar test was performed according the standard method (MacFaddin JF 1985). Ability of bacteria to deaminate Phenylalanine oxidatively converting it to phenyl-pyruvic acid is a property of *Proteus spp*. differentiating them from with the exception of rarely isolated *Rahnella aquatilis* and *Taturella ptyseos*. Phenyl-pyruvic acid is revealed by the presence of a characteristic greenish colour in the medium when it reacts with iron. Nowadays, this test and the urease production test, have great importance in the taxonomy of *Proteus spp*. *E. coli ATCC 25922* and *Enterobactor aerogenes ATCC 1304* were taken as positive and negative control strains respectively.

2.7.5 MR-VP test
Both MR and VP tests were done according to the Edwards and Ewing method (Edward PR and Ewing WH, 1962). Isolated bacterial colonies were inoculated on sterilized MR-VP media contains 0.7 gm% peptone mixture, 0.5 gm% Potassium Phosphate and 0.5 gm% Dextrose. Culture test tubes containing this media were allowed to grow for 3 days. After three days half volume of media was transferred to a clean tube. Five drops of 0.4 gm % solution of Methyl red was added to first half portion of culture for MR test. Positive reaction was indicated by red colour, where as negative reaction was taken as yellow colouration. *E. coli ATCC 25922* and *Enterobactor aerogenes ATCC 1304* were taken as positive and negative control strains for this test respectively.

In second half portion of culture 0.6 ml of 5% alpha naphthol in absolute alcohol and 0.2 ml of 40% sodium hydroxide was added. Culture tubes were shaken for 15 min. A positive test was indicated by development of a pink to red colour in media. *E. coli ATCC 25922* and *Enterobactor aerogenes ATCC 1304* were taken as positive and negative control strains.

2.7.6 Citrate Utilization
Citrate utilization test was performed according to the standard method (MacFaddin JF. 1985). Citrate agar media contains 0.1 gm% ammonium di-hydrogen phosphate, 0.5 gm% NaCl, 0.02 gm% MgSO₄, 0.008 gm% Bromothymol blue, 0.1 gm% di-potassium phosphate, 0.2 gm% Sodium citrate and 1.5 gm% Bacteriological agar. a single isolated colony and lightly streak the surface of the slant of citrate agar and allowed to incubate for 3-4 days. Change of medium colour from green to blue taken as positive reaction. No change of colour indicated by appearance of negative result. *P. vulgaris ATCC 29906* and *Enterobactor aerogenes ATCC 1304* strains were taken as negative and positive control respectively.

2.7.7 Urease test
Urease test for bacterial isolates was done according to the method of MacFaddin et al. (MacFaddin JF. 1985). Three test tubes were prepared with urea (20.0 gm/L), Agar (15.0 gm/L), NaCl (5.0 gm/L), KH₂PO₄ (2.0 gm/L) and phenol red (0.012 gm/L) in slant position and marked as sample, urea control and blank. Another one test tubes was prepared in same direction but without urea and marked as bacteria control. Isolates were streaked on the slant marked as sample and bacteria control. All these four test tubes were kept in 37°C for 20-22 hrs. Positive result indicates the colour of sample test tube to reddish pink or red. *Proteus vulgaris ATCC 29906* and *E. coli ATCC 25922* were taken as positive and negative control strains for the test.

2.7.8 Triple sugar Iron (TSI) tests
All the bacterial isolates were inoculated on sterilized Triple Sugar Iron Agar tubes ( Peptone mixture 2 gm%, Sucrose 1 gm%, Beef extract 0.3 gm%, Dextrose 0.1 gm%, Sodium thioussulphate 0.03 gm%, Lactose 1.0 gm%, Sodium Chloride 0.5 gm%, Yeast Extract 0.3 gm%, Ferrous Ammonium Citrate 0.025 gm% and Bacteriological Agar 1.2 gm%). Tubes were incubated overnight at 37°C. Change of colour of the media tubes were observed and interpreted according to Vanderzant et al. (Vanderzant et al. 1992). *Proteus vulgaris ATCC*
29906 and Shigella flexneri ATCC 12022 strains was considered as positive control negative control strains for the test.

2.7.9 Nitrate reduction test
Nitrate reduction tests were done according to standard method (Snell and Wright, 1941). Nitrate broth was prepared (casein Peptone 0.5 gm%, Beef Extract 0.3 gm%, Potassium Nitrate 0.1 gm%, Galactose 0.5 gm% and Disodium Phosphate 0.25 gm%) with Durham tubes in it. All the bacterial isolates were inoculated in to those tubes and incubated aerobically at 37°C for 24 hours. 0.8 gm% sulphanilic acid (dissolved in 5M acetic acid) and 0.6 gm% alpha naphthole (dissolved in 5M acetic acid) and Zinc dust were simultaneously added to those cultures. Appearance of red colour with gas production considered as positive result. A negative result was noted by no change of media colour. E. coli ATCC 25922 was taken as negative and Proteus vulgaris ATCC 29906 was taken as positive control strains respectively.

2.7.10 Gelatinase test
Gelatin hydrolysis test was performed according the standard method (Dash et al. 2012). Gelatin is solid at 20°C or less temperature and liquid at 35°C or higher temperature. Gelatin liquefies at about 28°C, so incubation is carried out at 35°C but kept in a refrigerator for about 2 hours before interpretation of the results. Proteus vulgaris ATCC 29906 and Staphylococcus aureus ATCC 25923 strains were taken as positive control and negative control respectively.

2.7.11 Carbohydrate fermentation tests
Isolates were also inoculated on Phenol red Dextrose broth (Casein peptone 1.0 gm%, NaCl 0.5 gm%, Dextrose 0.5 gm%, Phenol red 0.0018 gm %) with Durham tubes for glucose fermentation study. For Sucrose and Lactose fermentation isolates were inoculated on Phenol red Sucrose broth(Casein peptone 1.0 gm%, NaCl 0.5 gm%, Sucrose 0.5 gm%, and Phenol red 0.0018 gm%) and Phenol red Lactose broth (Casein peptone 1.0 gm%, NaCl 0.5 gm%, Lactose 0.5 gm%, and Phenol red 0.0018 gm%). For all three fermentation tests broth medium were incubated for 48 hours in an aerobic incubator. Positive result was noted by change of media colour from red to yellow and production of gas on Durham’s tube. E. coli ATCC 25922 and Shigella flexneri ATCC 12022 were taken as positive and negative control strains respectively (Murray PR et al. 1995).

2.8 Motility test
Motility test of isolates was performed according to the Tittsler et al. method (Tittsler et al. 1936). Briefly, properly sterilized semi solid agar tube (beef extract-0.3%, pancreatic digest of casein-1.0%, NaCl 0.5% and agar-0.4%) was prepared. Tubes were inoculated with a pure culture of isolates by stabbing the center of the column of medium to greater than half the depth and incubated for 24-48 hours at 37°C in an aerobic atmosphere. The growth of organisms was observed, whether it was through the line of inoculation or spread out from the inoculation. E. coli ATCC 25922 and Proteus vulgaris ATCC 29906 were taken as positive and negative control, respectively.

2.9 Antibiotic susceptibility testing of isolated P. vulgaris strains
2.9.1. Susceptibility testing by disc agar diffusion (DAD)
The disk diffusion method for antimicrobial susceptibility testing was determined by Kirby-Bauer technique (Bauer AW et al. 1966). The bacteria were taken from an overnight culture and freshly grown for 4 hours at approximately amount of $10^6$ CFU/ml. The bacteria were spread on Mueller-Hinton agar equivalently. About 6-mm size discs were used to observe antibiotic susceptibility patterns against 16 antibiotics Antibiotics were used as Ampicillin (10 µg), Amoxycillin Clavulanic acid (30 µg), Penicillin G (10 µg), Gentamycin (10 µg), Tetracycllin (30 µg), Erythromycin (15 µg), Amikacin (30 µg), kanamycin (30 µg), Streptomycin (10 µg), Cephotsaxime (30 µg), Chloramphenicol (30 µg), Oxacillin (1 µg), Imipenem (10 µg), Nalidixic acid 30 µg), Norfloxacin (10 µg), and Ciprofloxacin (5 µg). Antibiotic discs were obtained commercially from Himedia, India. The bacterial growth inhibition surrounding diameter of zone of the disc (including the disc) was measured by standard zone scale of Himedia, India and compared with the international standard for each drug disc. The Quality Control was performed using Proteus vulgaris ATCC 29906.
2.9.2 Determination of Minimum Inhibitory Concentration (MIC)
The MIC values of Penicillin, Ampicillin, Tetracyclin, Gentamycin, Erythromycin, Chloramphenicol, Streptomycin, Kanamycin, Cefotaxime, Nalidixic acid, Oxacillin, Norfloxacillin, Ciprofloxacin, Amikacin, and Imipenem, were determined by a broth dilution method using Mueller–Hinton broth (MHB), as recommended by the National Committee for Clinical Laboratory Standards (Buxton A et al. 1977). About 5 x 10⁴ cells in MHB were treated with different concentrations of antibiotics and shaken for 16 h at 37°C. The minimum concentration at which there was no visible turbidity was taken as the MIC of that antibiotic (Dash et al., 2012).

2.9.3. Determination of Minimum Bactericidal Concentration (MBC)
The MBC value of antibiotics was determined according to Dash et al., (Dash et al., 2012). This is an extension of the MIC Procedure. Antibiotics treated bacterial culture showing growth or no growth in the MIC tests were used for this test. Bacterial culture used for the MIC test were inoculated onto the Mueller–Hinton agar and incubated at 37°C for 24 hr. Microbial growth or death were ascertained via no growth on Mueller–Hinton agar plate. The minimal concentration of the antibiotic that produced total cell death is the MBC.

3. RESULTS

3.1 Biochemical tests of isolates
For identification of clinical isolates Standard conventional biochemical tests were done. Single colony isolation technique is used for separation and purification of bacterial isolates on CLED agar without Sodium chloride which exhibited to spread out swarming of Proteus vulgaris. From the study it was observed that, 59.53% isolates were Gram negative and 40.47% isolates were Gram positive; 95.23% of gram negative isolates are indole positive. 100% of indole positive isolates were Cathalase positive, MR positive, Nitrate reduction test positive, Carbohydrate fermentation (Glucose, Lactose, Sucrose) test positive and also positive for carbohydrate utilization with gas production on TSI. All Indole positive isolates were negative for Oxidase test, VP test, Urease test, Citrate test and Gelatinase test. It was also revealed that 100% of Indole positive isolates. On MacConkey agar w/o Crystal violet and EMB agar 100% of Indole positive isolates gave red-pink colour colony and green with metallic shine colony respectively. Among the 1260 clinical isolates, 36 isolates were confirmed to be uropathogenic Proteus vulgaris (Figure 1).

3.2 Antibiotic susceptibility testing
3.2.1 Antimicrobial susceptibility testing by disc agar diffusion (DAD)
Antibiotic resistance profile and pattern of multidrug resistance by DAD reveled that out of 36 P. vulgaris isolates 100% resistance property were found to penicillin G, ampicillin, more than 90% resistance to erythromycin, kanamycin, streptomycin, oxacillin and amoxicillin clavulanic acid; more than 80% resistant to gentamycin, tetracyclin, chloramphenicol, nalidixic Acid, ciprofloxacin; 75% resistance to norfloxacin; less than 70% or less resistance pattern was identified to cephotaxime. All 36 isolates were less than 50% sensitive to amikacin and imipenem (Figure 2).

3.2.2 MIC of antibiotics
The MIC values of Penicillin, Ampicillin, Tetracyclin, Gentamycin, Erythromycin, Chloramphenicol, Streptomycin, Kanamycin, Oxacillin, Norfloxacillin, Ciprofloxacin, Amikacin, and Imipenem for UPPV isolates were determined. In each set of experiment, bacterial control tubes showed no growth inhibitory effect of antibiotics. These MIC values were compared with the NCCLS breakpoints of Minimum Inhibitory concentration for P. vulgaris. It was observed that MIC values of penicillin G, erythromycin, streptomycin and oxacillin for 100% of isolated P. vulgaris strains; ampicillin and kanamycin for 90% of isolates; gentamycin and ciprofloxacin for 70% isolates; tetracyclin and norfloxacin for 60% isolates; cephotaxime, amikacin and chloramphenicol for...
40% UPPV strains were beyond the sensitive range (Table 1a, 1b).

3.2.3 MBC of antibiotics
The MBC values of all said antibiotics for P. vulgaris isolates were determined. In each set of experiment, bacterial control plates showed no growth. It was observed that MBC values of penicillin G, erythromycin, streptomycin and oxacillin for 100% of isolated P. vulgaris strains; ampicillin and kanamycin for 90% of isolates; gentamycin and ciprofloxacin for 70% isolates; tetracyclin and norfloxacin for 60% isolates; cephotoxime, amikacin and chloramphenicol for 40% P. vulgaris strains were beyond next two concentrations of MIC values (Table 1).

4. DISCUSSION
Gradually decreasing sensitivity of conventional and traditional antibiotics has become a serious threat to challenge the life-threatening infections caused by P. vulgaris. Previous studies revealed that intestinal microbial flora of the human being gradually filled up with the resistant genes of the normal and clinical bacteria resistance to the antimicrobials have increased over the years (Okeke IN. 2000). As a result an inevitable genetic response produces, which possess strong selective pressure imposed by antimicrobial chemotherapy. This drug pressure as well as wrong treatment policy increases drug resistance bacterial species. Then the plasmid resistant genes are spread out to other bacterial species (Dash et al. 2012). In our study, patient’s urine samples were collected from nearest district hospital. One thousand two hundred sixty (1260) clinical urine samples were collected for species identification and characterization by Gram staining and Standard Biochemical tests. In this study, 59.53% of clinical isolates were Gram negative and 40.47% isolates were Gram positive. Only Gram negative clinical isolates were selected in this study because it is well known that P. vulgaris is gram negative bacteria and thirty six (36) of the isolated organisms were identified as P. vulgaris. The gram negativity of the selected organism was due to the additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space. In Gram’s staining procedure, the crystal violet and iodine complex (CV – I) interacts with negatively charged outer layers bacterial cells and stained the cells purple. When a decolourizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the peptidoglycan layer is left exposed. The CV – I complexes are washed from the bacterial cell along with the outer membrane. After decolourization, the bacterial cell loses its purple colour and counterstained with positively charged safranin appears in pink or red colour (Barnes JH. 2003).

Generally Enterobacteriaeceae are Gram-negative bacteria which grow in the intestinal tract and urinary tract of humans and other animals. The IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) tests are commonly used to identify the enterobacterioaceae group included such as Proteus, Escherichia coli, Enterobacter and Klebsiella (Prescott LM. 1999 and Santo E et al. 2007). Our investigation revealed that from all the Gram-negative isolates only 36 isolates were phenylalanine (PPA) test positive. It is used to differentiate Proteus and Providencia species from other Enterobacteriaceae. The capacity to deaminate Phenylalanine oxidatively converting it to phenyl-pyruvic acid is a property of Proteus spp. differentiating them from other enterobacteria. Phenyl-pyruvic acid is revealed by the presence of a characteristic greenish colour in the medium when it reacts with iron. Nowadays, this test and the urease production test, have great importance in the taxonomy of Proteus spp. (Edward and Ewing, 1986). From the 36 PPA positive isolates about 94.44% were indole positive. It may possible when break down of the amino acid tryptophan to indole production by the tryptophanase enzyme of the isolates. A pink-colored complex is produced when Indole reacts with Kovac’s reagent (para-dimethylaminobenzaldehye). Indole positivity of those isolates differentiates them from most Klebsiella sp and Enterobacter sp. (MacFaddin JF. 2000). It also revealed that all PPA positive isolates was 100% MR test positive and VP test negative (Fig.1). Because, the PPA positive isolates oxidize glucose for energy by bacterial enzymes. The isolates fermented the glucose in MR media and decrease the pH level upto 4.4 and converted the methyl red indicator into red colour. Voges-
Proskauer was negative for all PPA positive isolates that may be due to lacking of production of acetyl methyl carbinal. MR positivity and VP negativity give strong support in favours of *P. vulgaris* (MacFaddin JF. 2000). It was observed that all PPA positive isolates shows 100% citrate test negativity. This may be unutilizationa of citrate by PPA positive isolates which produce carbon that reacts with Bromthymol blue and produce blue colour. This finding indicates it to *Proteus sp.* and *E.coli* (Kanungo S. 2009). All the isolates were oxidase negative that supports to Enterobacteriaee family. Oxidase negativity can happen when non availability of cytochrome c oxidase is not present in the bacteria enzyme that catalyze the transport of electrons between electron doners and redox dye which produce the deep purple colour (Farmer JJ et al. 1981). Urease test was done for the detection of urea hydrolysis ability of those clinical isolates. Positive results were observed for all indole positive isolates. Most of the urinary tract isolates are urase positive. In our study all clinical isolates were urase positive; this may be due to urease enzyme required for hydrolysis of urea to ammonia (MacFaddin JF. 2000). It was observed in this study that all isolated strains showed a positive result in nitrate reduction and positive result in gelatin liquefaction. Nitrate positivity of isolates may be due to use of nitrate (NO$_3^-$) as an external terminal electron acceptor in exchange of nitrite (NO$_2^-$) during anaerobic respiration (Fingold SM et al. 1974). All 36 isolates were gelatinase positive. The test is used to differentiate genera of gelatinase-producing -bacteria such *Serratia* and *Proteus* from other members of the family Enterobacteriaeeae (Jamil M et al. 2007). All 36 PPA positive isolates were 97.22% TSI Positive. The isolates showed that red to yellow slant and butt with production of hydrogen sulphide (H$_2$S) gas. This indicates the glucose and sucrose fermentation ability of those clinical isolates (Buxton A and Fraser G, 1977). It was also revealed that all the isolates fermented dextrose, sucrose, production of both acid and gas but not fermented lactose. This indicates another supports for carbohydrate fermentation ability of clinical isolates (Zinnah MA et al. 2007). All 36 isolates were grown on EMB agar and MacConkey agar w/o crystal violet for confirmation of *P. vulgaris* as differential media. Colourless colonies on EMB agar and MacConkey agar considered as positive identification of *Proteus sp.* Finally Indole positivity confirmed that it is *Proteus vulgaris* (O’Hara CM. et al. 2000). Satisfactory result on all biochemical tests and colony characteristic on differential agar it was confirmed that all thirty six (36) isolates were uropathogenic *P. vulgaris*. The clinically isolated *P. vulgaris* strains were newly named as PV, from PV- 1 to PV-36.

In this study, the result of disc agar diffusion revealed that the isolated *P. vulgaris* strains (PV-1 to PV-30) were 100% resistance to penicillin G, ampicillin, more than 90% resistance to amoxicillin clavulenic acid, erythromycin, kanamycine, streptomycine, oxacillin, more than 80% resistant to gentamycin, tetracycline, chloramphenicol, nalidixic Acid, ciprofloxacin; 75% resistance to norfloxacin; more than 60% resistance pattern was identified to cephotaxime,. All 36 isolates were more than 50% sensitive to amikacin and imipenem. From our study it was confirmed that all the isolates were multi drug resistant (MDR). In our study it revealed that the resistance to fluoroquinolones (ciprofloxacin, norfloxacin etc.) among clinical isolates was very alarming to all medical practitioners. Ciprofloxacin is a common and most effective drug regularly used for complicated UTIs. Imipenem and amikacin were more effective drug that we found to all isolates. This may be due to unavailability and expensiveness of imipenem in this area. Chloramphenicol, cephotaxime, amikacin and imipenem can be still prescribed for UTI as their resistance rate still under control. The high level antibiotics resistant among *P. vulgaris* isolates may be due to improper prescription policy, lack of drug guidelines and misuse of antibiotics. In many areas of India antibiotics can be easily available over the counter without prescription of registered medical practitioner. So, in India, there is a need for establishment of a new antibiotic intake policy immediately which will be strictly followed by the concerned authorities. From the last few years antibiotic resistance property of pathogenic *P. vulgaris* became a serious threat for human health. Multi drug resistance pattern became a great challenge for medical practioners to formulate antibiotic prescribing policies as well as to public health (DebMandal et al. 2011).
Figure 1

**Biochemical test of P vulgaris by standard biochemical methods**

![Biochemical test graph]

Figure 2

**Resistant pattern of antibiotics against P vulgaris by DAD method**, Here, Here, **AMP = Ampicillin (10 µg), AMC = Amoxycillin Clavulanic acid (30 µg), P = Penicillin (10 µg), GEN = Gentamycin (10 µg), E = Erythromycin (15 µg), K = Kanamycin (30 µg), S = Streptomycin (10 µg), CE = Cefotaxine (30 µg), NA = Nalidixic acid (30 µg), NX= Norfloxacillin (10 µg), OX = Oxacillin (1 µg), CIP = Ciprofloxacin (5 µg), TE = Tetracyclin (30 µg), C = Chloramphenicol (30 µg), AK = Amikacin (30 µg), IPM = Imipenem (10 µg)**

![Resistant pattern graph]
### Table 1

**Antimicrobial susceptibility testing by MIC, MBC**

| Strain | AMP μg/ml | AMC μg/ml | P μg/ml | GEN μg/ml | TE μg/ml | K μg/ml | S μg/ml | GEX μg/ml | C μg/ml | OX μg/ml | PFM μg/ml | NA μg/ml | NX μg/ml | CIP μg/ml |
|--------|-----------|-----------|---------|-----------|----------|---------|---------|-----------|---------|---------|-----------|----------|----------|----------|----------|
| PV-1   | 32        | 64        | 128     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-2   | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-3   | 32        | 64        | 128     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-4   | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-5   | 32        | 64        | 128     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-6   | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-7   | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-8   | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-9   | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-10  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-11  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-12  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-13  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-14  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-15  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-16  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-17  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| PV-18  | 64        | 128       | 256     | 256       | 128      | 256     | 128     | 256       | 128     | 256     | 128       | 256      | 128      | 256      | 128      |
| Strain | AMP µg/ml | AMC µg/ml | P µg/ml | GEN µg/ml | TE µg/ml | E µg/ml | AK µg/ml | K µg/ml | S µg/ml | CE µg/ml | C µg/ml | OX µg/ml | IPM µg/ml | NA µg/ml | NY µg/ml | CIP µg/ml |
|--------|-----------|-----------|---------|-----------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| PV-19  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-20  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-21  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-22  | 64        | 128       | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-23  | 64        | 128       | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-24  | 64        | 128       | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-25  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-26  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-27  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-28  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-29  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-30  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-31  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-32  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-33  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-34  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-35  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |
| PV-36  | 128       | 64        | 256     | 64        | 128     | 32      | 64      | 64      | 128     | 16      | 8       | 4       | 32      | 8       | 1       | 32      | 8       |

Here, AMP = Ampicillin, AMC = Amoxycillin Clavudanic acid, P = Penicillin, GEN = Gensamycin, E = Erythromycin, K = Kanamycin, S = Streptomycin, CE = Cefotaxine, NA = Nalidixic acid, NX = Norfloxacillin, OX = Oxacillin, CIP = Ciprofloxacin, TE = Tetracyclin, C = Chloramphenicol, AK = Amkacin, IPM = Imopenem, MIC = Minimum Inhibitory Concentration, MBC = Minimum Bacterial Concentration, PV = Proteus vulgaris
Table 2
Antimicrobial susceptibility testing by MIC, MBC

<table>
<thead>
<tr>
<th>Antibiotic sensitivity pattern</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP, P, GEN, E, K, S, CE, NA, NX, CIP</td>
<td>3 (8.33%)</td>
</tr>
<tr>
<td>AMP, AMC, P, GEN, TE, E, K, S, C, OX, IPM, NA, NX</td>
<td>3 (8.33%)</td>
</tr>
<tr>
<td>AMP, AMC, P, GEN, TE, E, K, S, C, CE, IPM, OX, CIP</td>
<td>2 (5.55%)</td>
</tr>
<tr>
<td>AMP, AMC, P, GEN, TE, E, AK, K, C, CE, OX, NA</td>
<td>1 (2.77%)</td>
</tr>
<tr>
<td>AMP, AMC, P, TE, AK, K, S, C, CE, OX, CIP</td>
<td>2 (5.55%)</td>
</tr>
<tr>
<td>AMP, AMC, P, TE, E, K, S, CE, OX, NA, NX, CIP</td>
<td>2 (5.55%)</td>
</tr>
<tr>
<td>AMP, AMC, P, GEN, E, AK, S, CE, OX, NA, CIP</td>
<td>1 (2.77%)</td>
</tr>
<tr>
<td>AMP, AMC, P, GEN, E, K, S, C, OX, NA, NX, CIP</td>
<td>2 (5.55%)</td>
</tr>
<tr>
<td>AMP, AMC, P, GEN, TE, E, AK, K, S, C, OX, IPM, NA, NX, CIP</td>
<td>5 (13.88%)</td>
</tr>
<tr>
<td>AMP, AMC, P, GEN, TE, E, K, S, CE, C, OX, IPM, NA, NX, CIP</td>
<td>7 (19.44%)</td>
</tr>
<tr>
<td>AMP, AMC, P, GEN, TE, E, AK, K, S, CE, C, OX, NA, NX, CIP</td>
<td>8 (22.22%)</td>
</tr>
</tbody>
</table>

Here, AMP = Ampicillin, AMC = Amoxycillin Clavulanic acid, P = Penicillin, GEN = Gentamycin, E = Erythromycin, K = Kanamycin, S = Streptomycin, CE = Cefotaxine, NA = Nalidixic acid, NX= Norfloxacinill, OX = Oxacillin, CIP = Ciprofloxacin, TE = Tetracyclin, C = Chloramphenicol, AK = Amikacin, IPM = Imipenem.

5. CONCLUSION

In our study, it may be concluded that all the thirty six (36) \textit{P. vulgaris} isolates were successfully characterized by biochemical techniques and high level of multidrug resistant pattern was found among all the isolates. All of the isolates were found resistant to more than ten antibiotics included in this study. Antibiotic resistance in common gram negative bacteria is becoming a big problem for the individuals admitted to the hospitals and the health care centers to treat for medical professionals. In that study all of the isolates showed multiple antibiotic resistance property. The isolates were found maximum resistance against ampicillin, penicillin G, erythromycin, streptomycin, cefotaxime, chloramphenicol and oxacillin whereas least resistance was found against imipenem and amikacin.

6. DECLARATION OF INTEREST

Authors declares that there are no conflict of interests.

7. ACKNOWLEDGEMENT

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ABBREVIATIONS

\textit{P. vulgaris} : Proteus vulgaris  
\textit{E. coli} : Escherichia coli  
\textit{E. faecalis} : Enterococcus faecalis  
\textit{S. aureus} : Staphylococcus aureus  
\textit{S. epidermidis} : Staphylococcus epidermidis  
UPPV : Uropathogenic \textit{Proteus vulgaris}  
CFU : Colony formation unit  
DAD : Disc agar diffusion  
MIC : Minimum Inhibitory Concentration  
MBC : Minimum Bactericidal Concentration  
MDR : Multi drug resistant
REFERENCES


