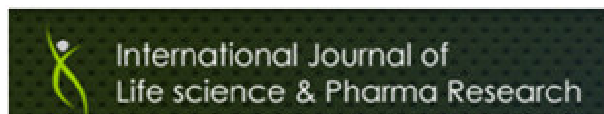
Research Article ISSN 2250-0480 VOL 5/ ISSUE 2/APRIL 2015



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

DEBASIS MANDAL [1], SANDEEP KUMAR DASH[1], BALARAM DAS[1], MANIDEEPA SENGUPTA [2], PRATIP KUMAR KUNDU [3] AND SOMENATH ROY [1*]

[1]Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore-721 102, West Bengal, India.
[2]Department of Microbiology, Midnapore Medical College & Hospital, 108, Chittaranjan Avenue, Kolkata - 700 073, West Bengal, India.
[3]Department of Protozoology, Calcutta School of Tropical Medicine, 108, Chittaranjan Avenue, Kolkata - 700 073, West Bengal, India.

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. H_2S production seen in 97.22% on Triple Sugar Iron (TSI) agar test respectively. 100% negativity was seen for Voges-Proskaue, 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.

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 nature and opportunistic human pathogen. It can found in intestine along with *E.*

coli and *Klebsiella* species, of which *E coli* is the predominant resident. It was also found that *E. coli* is responsible for approximately 90% of urinary tract infections and *P. vulgaris* infections are also seen (Dash et al. 2012 and Stamm WE et al. 1993). It was also noted that Proteus after the *E. coli*, rank third as the cause of infection of uncomplicated cystitis, phelonephritis, prostatitis and approximately 3% of nosocomial infections in the United States (Centers for Disease Control and Prevention, 1996). But it was also documented that *Proteus species* are also very much common in urinary tract infection after *E. coli*. Over the past two decades the genus *Proteus*, and in particular *Proteus vulgaris* has undergone a number of major taxonomic revisions. *Proteus mirabilis* causes 90% of *Proteus* mediated infections and can be considered a community-acquired infection (O'Hara CM et al. 2000). *P. vulgaris*, is an opportunistic pathogen and it was firstly described by Margit Luise Hauser in 1885. Hickman et al. found 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.

MATERIALS AND METHODS

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, Norfloxacin, Niprofloxacin, Amikacin and Imipenem were purchased from Himedia, India. Sodium Chloride (NaCl), Hydrogen Peroxide (H_2O_2), Sucrose, Lactose, Glucose, Galactose, Potassium dihydrogen phosphate (KH_2PO_4), Di potassium hydrogen phosphate (K_2HPO_4), 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.

Sample Collection and Transport

One thousand two hundred sixty (1260) UTI patent's 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).

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.

Quality control strains

S. aureus ATCC 29213, *Shigella flexneri* ATCC 12022 were obtained from Depertment fo Microbiology, Midnapore Medical College and Hospital, *S. 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 Depertment of Microbiology, Calcutta Medical college and Hospital. All the reference strains were kept and stored in agar slants at 4°C.

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.

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 37ºC 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).

Biochemical identification tests

Catalase test

For tube catalase test, 200µl of 3-6% hydrogen peroxide (H_2O_2) 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% H_2O_2 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).

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-pphenylenediamine 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.

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.

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 expection 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*. *P. vulgaris* ATCC *29906* and *E. coli* ATCC 25922 were taken as positive and negative control strains respectively.

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 napthanol 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.

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% $MgSO_4$, 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 media 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.

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), KH_2PO4 (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 37ºC 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.

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 thiosulphate 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 37ºC. 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.

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 37ºC 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.

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.

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).

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 37ºC 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.

Antibiotic susceptibility testing of isolated P. vulgaris strains

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), Tetraclyclin (30 µg), Erythromycin (15 µg), Amikacin (30 µg), kanamycin (30 µg), Streptomycin (10 µg), Cephotaxime (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.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC values of Penicillin, Ampicillin, Tetraclyclin, 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 10^4 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).

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.

RESULTS

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 Catalase 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).

Antibiotic susceptibility testing

Antimicrobial susceptibility testing by disc agar diffusion (DAD)

Antibiotic resistance profile and pattern of multi drug 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 clavulinic acid; more than 80% resistant to gentamycin, tetraclyclin, 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).

It was found that all the *Proteus vulgaris* isolates were 100% multidrug resistant. There are eleven types of resistance pattern were recognized among MDR strains. Among the 36 MDR strains 55.55% were resistant to 15 antibiotics, 13.88% to 13 antibiotics, 13.88% to 12 antibiotics, and 8.33% to 11, 8.33% to 10, (Table 2).

MIC of antibiotics

The MIC values of Penicillin, Ampicillin, Tetraclyclin, 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; tetraclyclin and norfloxacin for 60% isolates; cephotaxime, amikacin and chloramphenicol for 40% UPPV strains were beyond the sensitive range (Table 1a, 1b).

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; tetraclyclin and norfloxacin for 60% isolates; cephotaxime, amikacin and chloramphenicol for 40% *P. vulgaris* strains were beyond next two concentrations of MIC values (Table 1).

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 de-colourization, the bacterial cell loses its purple colour and counterstained with positively charged safranin appears in pink or red colour (Barnes JH. 2003).

Generally Enterobacteriaceae 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 carbinol. 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 unutilizataion 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 Enterobacteriaeae family. Oxidase negativity can happened when non availability of cytochrome c oxidase is not present in the bacteria enzyme that catalyze the transport of electrons between electron donors 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 urease positive. In our study all clinical isolates were urease 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 gilatinase positive. The test is used to differentiate genera of gelatinase-producing -bacteria such *Serratia* and *Proteus* from other members of the family Enterobacteriaceae (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_2S) 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 clavulinic acid, erythromycin, kanamycin, streptomycin, oxacillin, more than 80% resistant to gentamycin, tetraclycline, 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

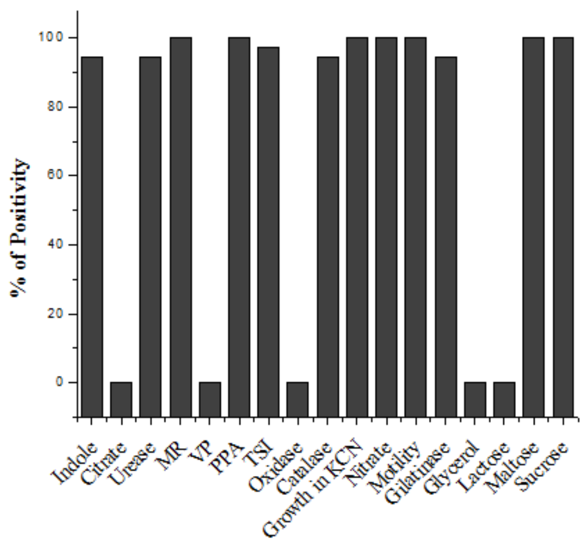


Figure 2
Resistant pattern of antibiotics against P vulgaris by DAD method, Here, Here, AMP = Ampicillin (10 µg), AMC = Amoxycillin Clavulanic acid (30 µg), P = Penicillin (10 µg), GEN = Gentamycin (10 µg), E = Erythromycin (15 µg), K = Kanamycin (30 µg), S = Streptomycin (10 µg), CE = Cefotaxine (30 µg), NA = Nalidixic acid (30 µg), NX= Norfloxacillin (10 µg), OX = Oxacillin (1 µg), CIP = Ciprofloxacin (5 µg), TE = Tetraclyclin (30 µg), C = Chloramphenicol (30 µg), AK = Amikacin (30 µg), IPM = Imipenem (10 µg)

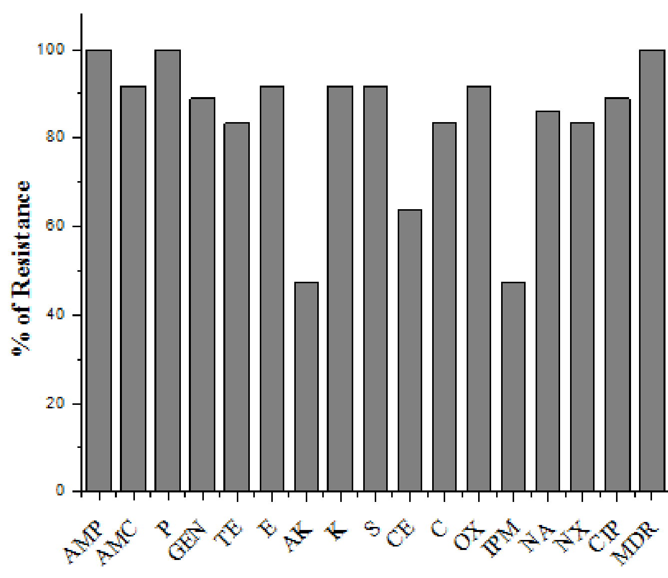


Table 1
Antimicrobial susceptibility testing by MIC, MBC

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	NX µg/ ml	CIP µg/ ml
	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC
PV- 1	32 64	128 256	128 256	64 128	32 64	16 32	8 16	32 64	2 4	1 2	8 16	8 16	1 2	32 64	32 64	4 8
PV-2	64 128	128 256	128 256	64 128	32 64	32 64	8 16	32 64	1 2	1 2	16 32	8 16	1 2	32 64	4 8	1 2
PV-3	32 64	32 64	64 128	16 32	64 128	32 64	8 16	16 32	2 4	1 2	16 32	8 16	2 4	32 64	16 32	1 2
PV-4	64 128	128 256	128 256	16 32	32 64	32 64	16 32	64 128	8 16	1 2	4 16	8 16	8 16	16 32	4 16	8 16
PV-5	32 64	128 256	64 128	32 64	64 128	32 64	16 32	8 16	1 2	1 2	4 16	8 16	1 2	8 16	4 8	1 2
PV-6	64 128	128 256	128 256	64 128	32 64	64 128	8 16	32 64	8 16	1 2	4 16	8 16	1 2	16 32	8 16	8 16
PV-7	64 128	128 256	128 256	8 16	16 32	32 64	8 16	32 64	8 16	1 2	4 16	8 16	1 2	8 16	4 16	8 16
PV-8	64 128	128 256	128 256	64 128	64 128	32 64	8 16	32 64	16 32	1 2	16 32	8 16	8 16	32 64	16 32	8 16
PV-9	64 128	128 256	128 256	64 128	64 128	32 64	8 16	64 128	8 16	1 2	16 32	8 16	1 2	16 32	4 16	8 16
PV-10	64 128	128 256	128 256	64 128	32 64	32 64	32 64	32 64	16 32	8 16	8 16	8 16	1 2	32 64	4 8	8 16
PV-11	64 128	128 256	128 256	64 128	32 64	64 128	32 64	32 64	16 32	8 16	16 32	8 16	1 2	32 64	32 64	8 16
PV-12	64 128	128 256	128 256	64 128	32 64	32 64	32 64	32 64	8 16	8 16	16 32	8 16	1 2	32 64	32 64	8 16
PV-13	64 128	128 256	128 256	64 128	16 32	64 128	32 64	32 64	16 32	8 16	16 32	4 8	1 2	16 32	32 64	8 16
PV-14	64 128	16 32	128 256	64 128	8 16	32 64	32 64	32 64	8 16	8 16	16 32	8 16	1 2	32 64	16 32	8 16
PV-15	64 128	8 16	128 256	64 128	32 64	32 64	32 64	32 64	16 32	8 16	32 64	4 8	1 2	32 64	8 16	8 16
PV-16	64 128	128 256	128 256	64 128	64 128	32 64	32 64	32 64	16 32	8 16	16 32	8 16	1 2	32 64	16 32	8 16
PV-17	64 128	8 16	64 128	64 128	16 32	32 64	32 64	32 64	8 16	1 2	32 64	4 16	1 2	32 64	32 64	16 32
PV-18	64 128	128 256	128 256	64 128	32 64	32 64	32 64	32 64	8 16	8 16	16 32	8 16	1 2	16 32	32 64	16 32

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	NX µg/ ml	CIP µg/ ml
	MIC MBC	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**
PV- 19	128 256	64 128	128 256	64 128	16 32	32 64	64 128	64 128	8 16	1 2	32 64	8 16	1 2	32 64	32 64	8 16
PV-20	128 256	64 128	128 256	64 128	32 32	32 64	64 128	64 128	8 16	4 8	32 64	8 16	1 2	32 64	32 64	4 8
PV-21	128 256	64 128	128 256	64 128	16 32	16 32	64 128	64 128	8 16	1 2	16 32	8 16	1 2	32 64	16 32	16 32
PV-22	64 128	64 128	128 256	64 128	16 32	16 32	32 64	32 64	8 16	8 16	32 64	8 16	1 2	32 64	16 32	8 16
PV-23	64 128	64 128	128 256	64 128	32 64	8 16	32 64	64 128	2 4	1 2	16 32	8 16	2 4	32 64	64 128	1 2
PV-24	64 128	64 128	128 256	64 128	64 128	32 64	64 128	64 128	8 16	1 2	16 32	8 16	1 2	32 64	32 64	16 32
PV-25	128 256	64 128	128 256	64 128	64 128	32 64	64 128	64 128	8 16	1 2	32 64	16 32	4 8	32 64	32 64	8 16
PV-26	128 256	64 128	128 256	64 128	64 128	64 128	64 128	64 128	8 16	1 2	32 64	16 32	1 2	32 64	32 64	16 32
PV-27	128 256	64 128	128 256	64 128	16 32	64 128	8 16	64 128	8 16	1 2	32 64	16 32	1 2	32 64	64 128	16 32
PV-28	128 256	64 128	128 256	64 128	32 64	32 64	32 64	64 128	8 16	8 16	64 128	16 32	1 2	32 64	32 64	8 16
PV-29	128 256	64 128	128 256	64 128	32 64	32 64	32 64	64 128	8 16	8 16	64 128	16 32	1 2	32 64	32 64	8 16
PV-30	128 256	64 128	128 256	64 128	32 64	32 64	8 16	32 64	8 16	1 2	64 128	8 16	1 2	32 64	32 64	8 16
PV-31	128 256	64 128	128 256	64 128	64 128	32 64	8 16	32 64	16 32	8 16	64 128	16 32	1 2	32 64	32 64	8 16
PV-32	128 256	64 128	128 256	64 128	32 64	32 64	16 32	32 64	16 32	8 16	64 128	16 32	2 4	32 64	32 64	8 16
PV-33	128 256	64 128	128 256	64 128	64 128	32 64	8 16	64 128	8 16	8 16	32 64	16 32	1 2	32 64	32 64	8 16
PV-34	128 256	64 128	128 256	64 128	64 128	32 64	32 32	64 128	8 16	8 16	32 64	16 32	1 2	32 64	32 64	16 32
PV-35	128 256	64 128	128 256	64 128	64 128	32 64	16 32	64 128	8 16	8 16	32 64	16 32	1 2	32 64	64 128	16 32
PV-36	128 256	64 128	128 256	64 128	64 128	32 64	64 128	128 256	8 16	8 16	32 64	32 64	1 2	32 64	32 64	8 16

Here, AMP = Ampicillin, AMC = Amoxycillin Clavulanic acid, P = Penicillin, GEN = Gentamycin, E = Erythromycin, K = Kanamycin, S = Streptomycin, CE = Cefotaxine, NA = Nalidixic acid, NX= Norfloxacillin, OX = Oxacillin, CIP = Ciprofloxacin, TE = Tetraclyclin, C = Chloramphenicol, AK = Amikacin, IPM = Imipenem, MIC = Minimum Inhibitory Concentration, MBC = Minimum Bactericidal Concentration, PV = Proteus vulgaris

Table 2
Antimicrobial susceptibility testing by MIC, MBC

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

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

CONCLUSION

In our study, it may be concluded that all the thirty six (36) *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, cephotaxime, chloramphenicol and oxacillin whereas least resistance was found against imipenem and amikacin.

DECLARATION OF INTEREST

Authors declares that there are no conflict of interests.

ACKNOWLEDGEMENT

The authors express gratefulness to Vidyasagar University, Midnapore, Mindapore Medical College & Hospital and Calcutta Medical College & hospital for providing required facilities to execute these studies.

ABBREVIATIONS

P. vulgaris : *Proteus vulgaris*
E. coli : *Escherichia coli*
E. faecalis : *Enterococcus faecalis*
S. aureus : *Staphylococcus aureus*
S. epidermidis : *Staphylococcus epidermidis*
UPPV : Uropathogenic *Proteus vulgaris*
CFU : Colony formation unit
DAD : Disc agar diffusion
MIC : Minimum Inhibitory Concentration
MBC : Minimum Bactericidal Concentration
MDR : Multi drug resistant
H_2O_2 : Hydrogen peroxide
KCl : Potassium chloride

K_2HPO_4 : Di potassium hydrogen phosphate
KH_2PO_4 : Potassium dihydrogen phosphate
LB : Luria broth
CLED : Cystine lactose electrolyte deficient
MHB : Mueller-Hinton broth
NA : Nutrient agar
NB : Nutrient broth
EMB : Eosin Methylene Blue
NaCl : Sodium chloride
NaOH : Sodium hydroxide
NCCLS : National Committee for Clinical Laboratory Standards
TSI : Triple sugar iron agar

REFERENCES

1. Barnes JH, Vaillancourt JP, Gross WB. Chapter 18. colibacillosis, In: Saif Y.M. et al. (eds.) : disease of Poultry, 11th edn. Lowa state university press, 2003; Ames, lowa, USA. PP. 631-652.
2. Bauer AW, Kirby WMM, Sherris JC, Turch M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966; vol 45: pp. 493-6.
3. Buxton A and Fraser G. Animal Microbiology. Escherichia coli, Blackwell Scientific Publications, Oxford, London, Edinburg, Melbourne. Vol. 1. pp. 92-102. 1977.
4. Caroline Mohr O'Hara, Frances W. Brenner and J. Michael Miller; Classification, Identification, and Clinical Significance of Proteus, Providencia, and Morganella. Clin Microbiol Rev. 2000. Vol. 13 no. 4, pp. 534–546.
5. Centers for Disease Control and Prevention, 1996. National nosocomial infections servelliance (NNIS) report, data summary October 1986 - April 1996, 1996. Am. J. Infect. Control. 24:381.
6. Duguid, J. P. Staining methods. In: Collee JG, Fraser AG, Marmion BP, Simmons A, editors. Mackie & McCartney Practical Medical Microbiology. 14th ed. Edinburgh: Churchill Livingstone, 1996. pp. 793-812.
7. Eaton et al. Standard methods for the examination of water and wastewater, 19th ed. American Public Health Association, 1995. Washington, DC.
8. Edwards PR and Ewing WH. Identification of Enterobacteriaceae. Burgess Publ. Co. Minneapolis, Minn. 1962.
9. Edwards and Ewing's identification of Enterobacteriaceae, 4th ed. Elsevier Science Publishing Co., Inc., 1986. New York, N.Y.
10. Farmer JJ, Fanning GR, Huntley-Carter GP, et al. Kluyvera, A new (redefined) genus in the family Enterobacteriaceae: identification of Kluyvera ascorbata sp. nov. and Kluyvera cryocrescens sp. nov. in clinical specimens. J Clin Microbiol. 1981; vol 13: pp. 919–33.
11. Finegold SM, Sutter VL, Ahebery HR, Rosenblatt JE. Isolation of Anaerobic Bacteria. Man Clin Micr Biol. 1974; pp. 365-375.
12. Hickman F. W., A. G. Steigerwalt, J. J. Farmer III, and D. J. Brenner. Identification of Proteus penneri sp. nov., formerly known as Proteus vulgaris indole negative or Proteus vulgaris biogroup. J. Clin. Microbiol. 1982; Vol 15: pp. 1097–1102.
13. Kanungo S. A Simplified Analysis of Different Escherichia coli Strains by Using RAPD Technique". Not. Bot. Hort. Agrobot. 2009; vol 37: pp 257-260.
14. K. Cohen-Nahum, L. Saidel-Odes, K. Riesenberg, F. Schlaeffer, A. Borer; Urinary Tract Infections Caused by Multi-Drug Resistant Proteus mirabilis: Risk Factors and Clinical Outcomes. 2010; Infection; 38: pp. 41–46.
15. Manisha Deb Mandal, Shyamapada Mandal and Nishith Kumar Pal, Antibiotic Resistance Prevalence and Pattern in Environmental Bacterial Isolates. The Open Antimicrobial Agents Journal. 2011; vol 3, pp. 45-52.
16. MacFaddin. J. F. Media for isolation cultivation-identification maintenance of medical bacteria. Wikins, Baltimore, MD. 1985; vol. 1. pp. 634-636.

17. MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. Philadelphia, Lippincott Williams & Wilkins. 2000; 3rd ed. pp 451-453.
18. Mehwish Jamil, Saira Bashir, Mashkoor Mohsin, Ayesha Tariq, Aasia Bashir,Asma Haque, Yasra Sarwar, Aamir Ali, Abdul Haque; Differentiation Of Common Gram Negative Pathogens By Pcr-Ribotyping. Pak J Med Sci. 2007; Vol. 23 pp. 233-237.
19. Murray PR., Baron EJ, Pfaller MA, Tenover FC, Yolken RH . Manual of clinical microbiology. American Society for Microbiology.1995; Washington DC. 6th edition.
20. NCCLS. Methods for Dilution Antimicrobial Susceptibility Tests for Identification of Medical Bacteria. Philadelphia; Lippincott Williams & Wilkins. 2000; 3rd ed. pp-451-453.
21. Okeke IN, Susan T. Fayinka, and Adebayo Lamikanra; Antibiotic Resistance in Escherichia coli from Nigerian Students. 1986–1998. Emerging Infectious Diseases. 2000; Vol. 6, No. 4.
22. Prescott LM, Harley JP, Klein DA. Isolation of pure bacterial cultures from specimens". Microbiology International (4th edition) WCB McGraw's Hill Companies Boston. 1999; pp. 714-796.
23. Robert J. Broomfield, Sheridan D. Morgan, Azhar Khan and David J. Stickler. Crystalline bacterial biofilm formation on urinary catheters by urease-producing urinary tract pathogens: a simple method of control. Journal of Medical Microbiology. 2009; vol 58, pp. 1367-1375.
24. Sandeep Kumar Dash, Subhankari Prasad Chakraborty, Debasis Mandal And Somenath Roy; Isolation And Characterization Of Multi Drug Resistant Uropathogenic Escherichia Coli From Urine Sample Of Urinary Tract Infected Patients. International Journal of Life Science & Pharma Research. 2012; Vol 2, pp. 2250-0480.
25. Santo E, Salvador MM and Marin JM. Multidrug-Resistant Urinary Tract Isolates of Escherichia coli from Ribeirão Preto, São Paulo, Brazil. The Brazilian Journal of Infectious Diseases. 2007; vol 11: pp. 575-578.
26. Santo Fuchs, P.C., A. L. Barry, S. D. Brown and the AST Survillance Group. Survey of antimicrobial activity of four commonly used third generation cephalosporins tasted against recent bacterial isolates from ten American medical centers and assement of disk diffusion test performance. Diagn. Microbiol. Infection. . 1996; Dis. Vol 24: pp. 213-219.
27. Sarah M. Macleod and David J. Stickler. Species interactions in mixed-community crystalline biofilms on urinary catheters. Journal of Medical Microbiology. 2007; vol 56, pp. 1549-1557.
28. Snell and Wright. A microbiologic method for the determination of nicotinic acid. J Biol. Chem. 1941; vol 13: pp. 675.
29. Snell et al.; editors. Quality Assurance Principles and Practice in the Microbiology Laboratory. Public Health Laboratory Service. 1999; London: pp. 147-148.
30. Stamm WE, Hooton TM. The management of urinary tract intection. N Engl J Med. 1993; vol 329: pp. 1328-1334.
31. Stickler D, Morris N, Moreno MC, Sabbuba N. Studies on the formation of crystalline bacterial biofilms on urethral catheters. Eur J Clin Microbiol Infect Dis. 1998;17(9):649-52.
32. Tittsler RP, Sandholzer LA. The use of semi-solid agar for the detection of bacterial motility. J. Bacteriol; vol 31: pp. 575-580. 1936
33. Vanderzant et al. Conpendium of methods for the microbiological examination of foods. 3rd ed. American Public Health Association. Washington DC. 1992.
34. Zinnah MA, Bari MR, Islam MT, Hossain MT, Rahman MT, Haque MH, Babu SAM, Ruma RP Islam MA. Characterization Of Escherichia Coli Isolated From Samples Of Different Biological And Environmental Sources. Bang. J Vet Med. 2007; vol 5: pp. 25–32.