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"Phenotypic and Genotypic characterization Of Extended Spectrum Beta lactamase (ESBL) Producers among proteus isolates from patients with wound infection in tertiary care hospital"

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ABSTRACT:

Background:

Proteus species are frequently recovered from infected wounds. They contaminate wounds and thus cause infections. Microorganisms infecting wounds can multiply and colonize in the wound, resulting in host tissue damage. The Extended spectrum β-lactamases (ESBLs) production is one of the most essential Mechanism of drug resistance in Proteus species.

Aim & objectives:

The purpose of the study was Phenotypic and genotypic detection of extended spectrum β - lactamases production in Proteus isolated from wound infection samples.

Materials and methods:

This study was conductedfrom august 2022 to October 2022 at Department of Microbiology, Govt. Stanley Medical College& hospital in Chennai. A total of 480 Pus andwound swabs were collected from patients with wound infection, admitted in surgery ward. All the swab and pus samples collected were tested for the direct microscopy, culture, biochemical reaction by standard microbiological techniques.Proteus isolates was identified using differential media, biochemical tests (catalase, oxidase, IMViC, urease, and sugar fermentation) and antimicrobial susceptibility testing was done. ESBL production was confirmed by combined disc diffusion method by phenotypically. Then ESBL producing isolates were tested for presence of the blaSHV and blaCTX-M genes by polymerase chain reaction.

Results:

Among the 480 various wound swab/pus samples processed, 86 Proteus species were isolated. Among 86 Proteus isolates 60 (69.8%) were isolated from male patients and 26 (30.2%). Of the 86 isolates, 54 (63%) were identified as Proteus mirabilis and Proteus vulgaris 32 (37%). The organism resistant pattern to various antibiotics is as follows: Cotrimoxazole (67.5%), Cefazolin (62%), Ciprofloxacin (53.5%), Amoxicillin–clavulunate (53%) and Gentamycin (51%). Least resistance were shown to Piperacillin- tazobactem (17.4%), Meropenem (10%), Imipenem (15%), Cefepime (25.5%). Out of 86 proteus isolates 26 (30%) isolates were ESBL producers, as determined phenotypically by combined disc diffusion test. Genotypically; ESBLs genes were detected and the most prevalent ESBL resistance gene was CTX-M (30.8%) followed by SHV (11.5%).

Conclusion:

Monitoring of Extended spectrum β-lactamase producing P. mirabilis is very important because of its high prevalence among wound infections. It is necessary to increase awareness for clinicians and enhancing laboratories testing to rapidly identify these resistant organisms.This in turn is required to minimize the spread of these bacteria and helps clinician to select appropriate antibiotics.

KEY WORDS: ESBL resistant, SHV, CTX-M, Proteus spp.

I. INTRODUCTION:

Proteus species are Gram negative bacteria, belong to the Enterobacteriaceae family. There are several species of Proteus including Proteus mirabilis, P. vulgaris, P. penneri, P. hauseri, P. myxofaciens and P. rettgeri.Proteus species are widespread in the environment and make up part of the normal flora of the human gastrointestinal tract. They cause a variety ofcommunity and hospital-acquired illnesses, including urinary tract infection,wound infection, andbacteremia. Various studies show prevalence of Proteus species in wound infection as 14.7% Nithya Gomatheswari S et al, 26.8% R. M. Mordi et al, 13.3% Umbreen Zafar et al and 4.5% M. H. Bhalchandra et al. [3, 9,17,18] The severity of the disease caused by Proteus depends on the virulent characteristics of the organisms, including biofilm production, urease enzyme production and resistance to different classes of antibiotics. [1]

Proteus species are frequently isolated from infected wounds. Microorganisms infecting

wounds can multiply and colonize in the wound, resulting in host tissue damage. [17] A major problem in wound infections is the increasingly antimicrobial resistance has been reported for this genus and thepredominant mechanism for resistance to β-lactam antibiotics is by the synthesis of β-lactamases. Among the β-lactamases, the production of extended spectrum β-lactamases (ESBLs)is most common. ESBLs are plasmid-mediated β-lactamases that are capable of efficientlyhydrolyzing penicillin, narrow and broad-spectrum cephalosporins andmonobactams and are inhibited by β-lactamase inhibitors (e.g., clavulanicacid, sulbactam,and tazobactam). [3]

The presence of ESBL plasmids leads to their spread through horizontal gene transfer across bacteria of the same and other species. The prevalence of ESBL-producing isolates in each area are affected by a variety of parameters, including species, geographic region, hospital/ward, patient group, and kind of infection, as well as higher antibiotic usage. [4]Understanding the local distribution of antimicrobial resistance pattern and the prevalence of ESBL among Proteus species is critical for assisting physicians in their treatment decisions. [5,6] This study was aimed to identify ESBL producers and their associated resistance genes (CTX-M, SHV) in Proteus species isolates from wound infection by both phenotypic and genotypic methods, respectively.

II. MATERIALS AND METHODS:

Bacterial isolation and identification:

The study was conducted in the Department of Microbiology, Govt. Stanley Medical College and Hospital, Chennai for a period of 3 months, (August 2022 to October 2022) after getting Institutional Ethical Committee approval. Patients 18yrs and above who are admitted in general surgery ward with clinical sign/symptoms suggestive of wound infection during the study period were included. Appropriate samples were collected under sterile techniques. The samples were processed by standard microbiological techniques. Clinical samples were inoculated onto Nutrient agar, Blood agar, and MacConkey agar and incubated at 37°C for 18 to 24 hours. After the period ofincubation, Proteus species was identified by its morphology as swarming greyish white colonies on Blood agar, Nutrient agar, and non-lactose fermenting colonies on Mac-Conkey agar. Further identification of Proteus genus was done by phenylalanine deaminase production and speciation was done by biochemical reactions like H2S gas production in TSI, urease hydrolysis and indole production tests. [7,8]

Antimicrobial susceptibility test:

For antimicrobial susceptibility testing, bacterial suspensions of the isolate is prepared and matched with 0.5 McFarland standard for lawn culture on Muller-Hinton agar by modified Kirby Bauer disc diffusion method. Escherichia coli American type culture collection (ATCC) 25922 was used as control, and the results were interpreted as per CLSI guidelines 2022.

Standard antibiotics like, commercially available discsof amoxicillin-clavulanic acid (20/10 µg), piperacillin-tazobactam (100/10 µg), cefazolin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), amikacin (30 µg), cotrimoxazole (25 µg), gentamicin (10 µg), ciprofloxacin (5 µg), imipenem (10 µg) and meropenem (10 µg) were obtained from Himedia, Mumbai India.

Screening for extended spectrum β-lactamases production:

As per the CLSI recommendation, Proteusisolates showing zone of inhibition ≤22 mm for ceftazidime and ≤25 mm for cefotaxime by disc diffusion method were considered potential ESBL producers. These isolates were further tested for phenotypic confirmatory test for ESBL production was done by combined disc diffusion test. (CLSI 2022).

Confirmatory combined disc diffusion test: (CLSI 2022)

A disc of Ceftazidime 30µg and Ceftazidime-clavulanate 30µg/10µg were placed at a distance of about 24mm and a disc of Cefotaxime 30µg and Cefotaxime clavulanate 30µg/10µg were placed at a distance of about 24mm on Mueller Hinton agar with the lawn culture of isolated Proteus species and incubated at 37°C for 18 to 24 hours. After 24hrs of incubation, those isolates which shows > 5 mm increase in the zone diameter of either of the antimicrobial agent tested in combination with clavulanate compared to the zone diameter of the agent when tested alone was confirmed as ESBL producers, phenotypically. [8] (Fig-2) The phenotypically confirmed proteus ESBL isolates were further evaluated for the presence of ESBL associated genes blaCTX-M and blaSHV by polymerase chain reaction.

Detection of blaCTX-M and blaSHV genes by Polymerase Chain Reaction:

(i)DNA extraction:

DNA extraction was done by using IGB Bacterial DNA extraction Kit (column based).

Inoculate a single colony in 1ml of Luria Bertani broth and incubate at 37 ˚C overnight. After incubation, check for the growth of the culture and centrifuge at 8000 rpm for 5min at room temperature to pellet down. After centrifugation, discard the growth medium. Add 200µl re-suspension buffer to the pellet and re-suspend thoroughly by pipette mixing followed by Adding 200µl lysis buffermix by pipette mixing. Then to this add 5µl proteinase K, vortex well (5 to 10seconds) and incubate at 56℃ for 10min. After incubation, add 200µl 100% Ethanol and mix well. Transfer the mixture immediately to spin column and centrifuge at 8000rpm for 2min, discard the flow through. Add 500µl of wash buffer 1 and centrifuge at 8000rpm for 1min, discard the flow through. Then Add 500µl of wash buffer 2 and centrifuge at 8000rpm for 1min, discard the flow through. Dry spin for 8,000rpm for 2min. Transfer the spin column to new sterile 1.5ml tube. Add 40µl of pre-warmed elution buffer to spin column incubate at room temperature for 5min followed by Centrifuge at 8,000rpm for 1min. Transfer the gDNA to new sterile 1.5ml tube. Store the extracted DNA at -20℃ for further downstream application.

(ii) PCR:

A PCR targeting blactxM and blaSHV genes was performed for the Proteus isolate with 20µL reaction volume consisting of IGB balctxM and blaSHV primers (ImmuGenix Biosciences, India), 10× PCR buffer ,10 mM of dNTP mix, 1 units Taq DNA, DNA template (0.1-1 µg of DNA) and PCR grade water. The PCR amplification was performed in Veriti 96-Well Thermal Cycler (Applied Biosystems, USA) with specific primers and steps involved in PCR were mentioned in table-2. After PCR, the amplicons were resolved along with DNA markers in 1% agarose with ethidium bromide (10 mg/mL) by gel electrophoresis for ~15min at 135 V using Mupid-exU system (Takara, Japan) and gel was analysed by BioGlow UV Transilluminators (Crystal Technology, USA).

StatisticalAnalysis:

The results/ data were collected, documented and statistical interpretation was done usingSPSSsoftware.

III. RESULT:

Out of 480 samples received from patients suspected with wound infection, 396 (82.5%) samples yielded positive culture and there was no growth in 84 (17.5%) samples. Among 396 culture positive samples 21.7% (86/396) were positive for Proteus species while 78.3% were negative for proteus. Most of the Proteus species were isolated from pus samples 67.4% (58/86) followed by wound swab 32.6% (28/86). Among 86 Proteus isolates 60 (69.8%) were isolated from male patients and 26 (30.2%) were isolated from female patients. Two Proteus species isolated from 86 specimens were, P. mirabilis 62.8% (54/86) and P. vulgaris 37.2% (32/86). The clinical samples were received from age group 18-30 (44%), followed by 31-45[34%] and 46-60 (22%).

The antimicrobial resistant patterns of Proteus isolates in our study were evaluated by the disc diffusion method.Maximum resistance was seen with Cotrimoxazole (67.5%), Cefazolin (62%), Ciprofloxacin (53.5%), Amoxicillin–clavulunate (53%) and Gentamycin (51%). Least resistance was shown to Piperacillin- tazobactem (17.4%), Meropenem (10%), Imipenem (15%) and Cefepime (25.5%).(Table-2)

Among the 86 Proteus isolates included in the study, 29 isolates were screened positive for ESBL production. Out of 29 screened-positive isolates, 26 isolates were confirmed as ESBL producers by combined disc diffusion test. (Fig-3)

The 26 phenotypically confirmed ESBL producers were genotyped for the presence of associated resistant genes by PCR. The molecular characterization revealed that 30.8% (8/26) isolates possessed the blaCTX-M gene, whereas 11.5% (3/26) isolates possessed blaSHV gene. [Fig- 4&5] No isolates possessed both CTX-M an SHV genes together.The present study also showed 57.7% of phenotypically confirmed resistant strain did not show the presence of either or both of CTX-M and SHV genes.

IV. DISCUSSION:

The present study was carried out from August 2022 to October 2022 in the Department of Microbiology, Govt. Stanley Medical College & Hospital. A total of 480 wound swabs or pus samples were received, out of which 396 (82.5%) were culture positive and 84 (17.5%) were culture negative no growth (Fig -1). In our study, out of 396 culture positive for wound infection, Proteus species was 21.7% (86/396) which was accordance with 26.8% in Mordi R.M. et al, 28.5% in Snega Priya P et al, 13.3% in Umbreen Zafar et al and 14.7% in Nithya Gomatheswari S et al. [9,10,14,17] Whereas low prevalence noted in studies done by Bhalchandra M. H. et al in 4.5%

and 5.4% in Pal N et al. [3,18]The difference in the Proteus species prevalence rates may be related to their distribution in the various environments, type of wound involved and colonizing organisms is different from one geographical to another area.

This study shows male preponderance (69.8%) as compared to female (30.2%). It was noted in similar studies by Snega Priya P et al, Nithya Gomatheswari S et al and Raghav Rao et al which shows highest occurrence in males 65%, 59.06 and 58.82% in, respectively. [9,13,14] Most of the Proteus species were isolated from pus samples (67.4%) compared to wound swab (32.6%) (table-1); it is correlated with similar study by Snega Priya P et al and Pal N et al. [3,14] The male preponderance may be due to, males are commonly involved in our door works and exposure to environmental organisms compare to females. Pus samples yield higher rate of organismisolation compare to wound swab, this may be due to sample quantity and organism load is higher in pus samples compare to wound swab.

In the present study, P. mirabilis was the most common 62.8% (54/86) species isolated in wound infection samples, followed by P. vulgaris 37.2% (32/86)(table-1), which was similar to other studies done by Snega Priya P et al and Pal N et al. [3,14] P. mirabilis is the most common proteus species in the gastrointestinal tract as commensal and also present in the environment (soil) this may be connected to its higher incidence of wound infection.

In the present study, Maximum resistance was seen with Cotrimoxazole (67.5%), Cefazolin (62%), Amoxicillin– clavulunate (53%) and Gentamycin (51%), (Fig-2) which is similar to the rate in a study conducted by Snega priya P et al, Nithya Gomatheswari S et al and Pal N et al. [3,9,14] This higher resistance to commonly used antibiotics may be due to irrational use of antibiotics without proper culture and sensitivity testing and lack of awareness about organism resistance nature.

In the present study, The Extended Spectrum Beta Lactamase (ESBL) producers among Proteus isolates was found 30.2% (26/86). This was in accordance with studies done by Rasha Barwa et al (28.3%) and Snega Priya P et al (39%). [6,14] Whereas other studies done by Nithya Gomatheswari S et al, Maninder Kaur et al and Pal N et al were found ESBL producer higher range about 49%, 45.8% and 88% respectively. [3.9,16] The difference in the ESBL prevalence rates may be related to presence of high virulence nature and resistance genes in colonizing organisms in the environment and different from one geographical to another area.

In the present study, among the 26 ESBL producing Proteus isolates, blaCTX-M gene was detected in 8 isolates (30.8%), followed by the blaSHV gene detected in 3 isolates (11.5%). [Table-3]This finding accordance with study done by Akujobi C N et al and Maninder Kaur et al which shows most prevalent gene was CTX-M followed by SHV gene. [11,16] But this finding not correlate with study done by Ramadan Hassana et al which shows most prevalent gene was TEM, followed by CTX-M and SHV. This difference may be due to organisms having resistance genes is varies from one geographical area to another area. blaSHV and blaCTX-M genes are selected for the present study because of data related to these two genes was limited.

The present study also showed 57.7% of the phenotypically confirmed resistant strain did not show the presence of any CTX-M and SHV genes. It may be due to the presence of other genes like TEM, VEB and PER in the clinical specimens, which was not screened in this study. The prevalence of genes also varies from one geographical area to another area.

V. CONCLUSION:

The pathogenesis of Proteus species in wound infection and its antimicrobial sensitivity are major problems worldwide. β-lactamase production is an important cause of multiple and extensive drug resistance. The ESBL-producing organisms are tremendously increasing in the current scenario and are negatively affecting the patient's prognosis. Incorrect identification of antibiotic resistance may lead to inappropriate antibiotic prescription and treatment failure, only screening test will not be sufficient for detection of antibiotic resistance. Hence, reliable phenotypic confirmatory such as combined disc diffusion test should be done in routine microbiology laboratory, to identify the resistance among Proteus species. Phenotypic tests for ESBL detection only confirm whether an ESBL is produced but cannot detect the ESBL subtype and cannot detect those genes whose expression is hidden or masked. Therefore, the genotypic method is suggested as the method of choice for detection of ESBL-producing strains of proteus species. Molecular methods are sensitive, but they are expensive and require specialized

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equipment and expertise. Furthermore, genotypic methods can only detect those genes with known sequences. Hence, Phenotypic tests need to be evaluated periodically and methods of ESBL detection should be improved. This in turn is required to identify and to minimize the spread of these resistant bacteria and helps clinician to select appropriate antibiotics.

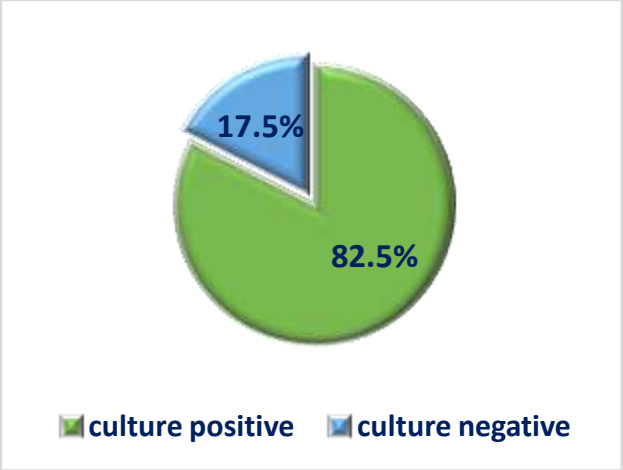

Fig 1- Culture positivity among wound infection samples

Out of 480 wound specimens**,** 396 (82.5%) samples yielded positive culture and there was negative culture growth in 84 (17.5%) samples.

Table 1: Proteus species isolated from clinical samples in the study (n=86)

S.No.	Type of Sample	Proteusmirabilis	Proteusvulgaris	Total (n=86)
1	Pus	36	22	58 (67.4%)
2	Wound swab	18	10	28 (32.6%)
	Total	54 (62.8%)	32 (37.2%)	86 (100%)

Most of the Proteus species were isolated from pus samples 67.4% (58/86) followed by wound swab 32.6% (28/86). Out of 86 Proteus isolates, P. mirabilis was 62.8% (54/86) and P. vulgaris was 37.2% (32/86).

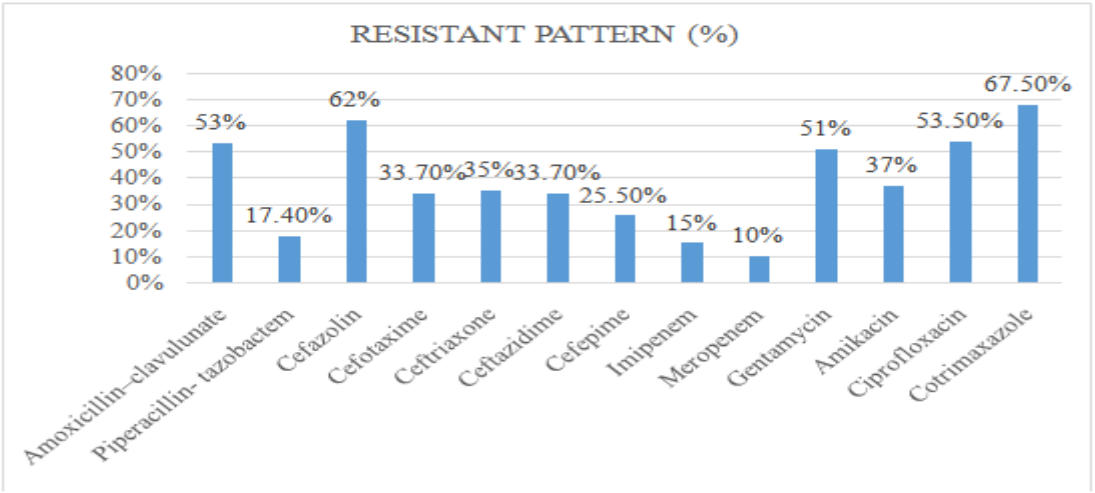

Fig -2Antibiotics resistant pattern of Proteus strains in this study [n=86]

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Fig-3: Phenotypic confirmation of ESBL isolates by combined disc diffusion test. CEC: ceftazidime plus clavulanic acid (30/10 mcg); CAC: Cefotaxime +clavulanic acid (30/10 µg); CTX: Cefotaxime (30 µg); CAZ: Ceftazidime (30 µg).

Table 2: Specific primers used for ESBL genes detection and optimum time & temperature for PCR

S.No	Gene	Primer Sequence	PCR Condition	Product Size
1	CTX-M	F- 5'-ATTCCRGGCGAYCCGCGT-3' R- 5'-ACCGCGATATCGTTGGTG-3'	Initial denaturation at 95°C for 3 min followed by 35 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 45 s, and with final extension at 72°C for 7 min.	227bp
2	SHV	F- 5' TCAGCGAAAAACACCTTG 3' R- 5'GCTGCGGGCCGGATAACG-3'	Initial denaturation at 94 °C for 5 min; followed by 30 cycles with 94 °C for 1min, 60 °C for 2 min, and 72°C for 1 min; final extension:72 °C for 10 min.	475bp

Table – 3 Associated resistant ESBL genes in our study isolates (n=26)

S.no	Name of the resistant genes	No. of isolates	Percentage %
1	blaSHV positive	8	30.8%
2	blaCTX-M positive	3	11.5%
3	Both genes positive	0	0%
4	Both genes negative	15	57.7%

Fig-4: PCR finding of blaCTX-M gene by gel electrophoresis

Detection of blaCTX-M gene using PCR technique. 1-9 isolates, lane 10: positive control, (227bp)N denotes Non-Template Control, M- Marker

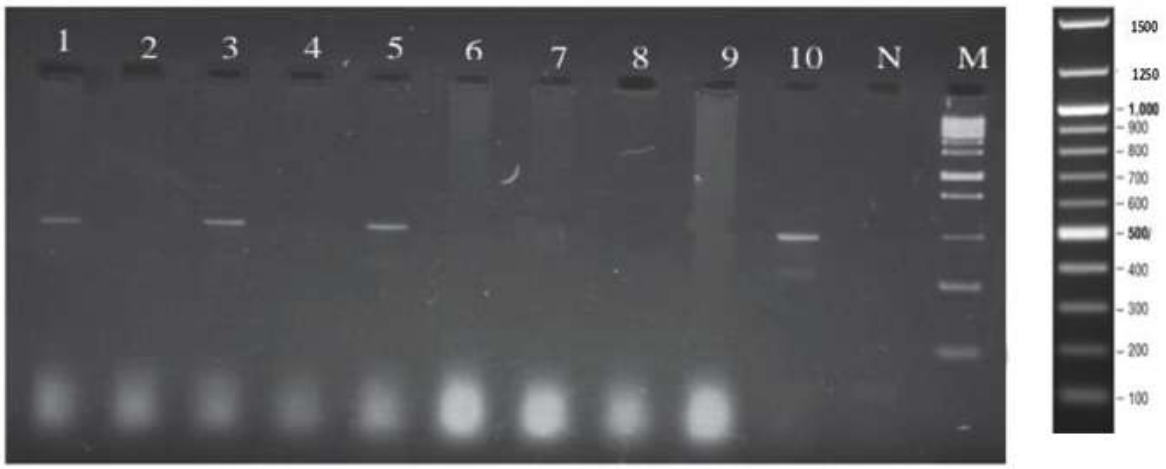

Fig-5: PCR finding of blaSHV gene by gel electrophoresis

Detection of blaSHV gene using PCR technique. 1-9 isolates, lane 10: positive control, (475bp)N denotes Non-Template Control, M- Marker

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