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Extended Spectrum β-Lactamase (ESBL) and Metallo β-Lactamase (MBL) Production in Gram-Negative Bacteria isolated from Urinary Tract Infection Patients.

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ABSTRACT

Background: The rate of infections caused by Morganella species is reported to be affected by difficulties in their isolation. Enterobacter, species, Citrobacter freundii, Serratia marcescens, and Morganella morganii are the major pathogens involved in hospital acquired infections. All of them are extensively reported in the infections of urinary tract, respiratory tract, cutaneous disorders, catheter associated infections and infections of the central nervous system.

Objective: To investigate the frequency of Extended Spectrum β- Lactamase producing bacteria in Urinary Tract Infection.

Material and Methods: A cross-sectional study of 200 urine samples was collected from the outdoor patient departments of Khyber Teaching Hospital Peshawar through consecutive sampling technique during April, 2019 to June 2019. The urine samples were collected and then cultured on selective media i.e., Cysteine Lactose Electrolyte Deficient Agar (CLED) and on MacConkey agar plates. Isolates were identified by conventional morphological and biochemical tests while antibiotic susceptibility was analyzed by "Agar disc diffusion method" using different antibiotics and their zone of inhibition was measured.

Results: Of a total 200 samples processed, 125 (62.5%) isolates, including 67 (53.6%) females, tested positive for the growth of Gram-negative bacteria. Among the identified species, 76 (60.8%) isolates produced ESBLs, with Escherichia coli accounting for 32.6%, Enterobacter for 23.2%, and Morganella species for 12.0%. Moreover, 77 (61.6%) of the isolates were found to be MBL positive, with 30 (24.0%) of them being E. coli, 28 (22.4%) being Enterobacter, and 19 (15.2%) being Morganella spp. The isolates formed a zone of inhibition like a clover leaf and demonstrated a sensitivity of 85 (68%) to antibiotics in the carbapenem class, such as imipenem and meropenem. Cefoparazone/Sulbactam 80 (64.0%), Ofloxacin 79 (63.2%), and Amikacin 78 (62.4%) were the next three antibiotics with high sensitivity. Ampicillin 106 (84.8%) showed the highest resistance, followed by Nitrofurantoin 84 (67.2%) and Ceftriaxone 79 (63.2%), in that order.

Conclusion: The study indicated that ESBL-producing bacteria were present frequently among UTI patients. Most of the isolates exhibited strong carbapenem class antibiotic sensitivity. The findings of this study may contribute to the formulation of tactics to lower the incidence of ESBL-producing UTI.

Keywords: Antibiotics resistance, Gram Negative bacteria, Plasmid-mediated enzymes, UTI.

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INTRODUCTION

Gram-negative pathogens known as Morganella species belong to the Protea group in the Enterobacteriaceae family. The two subtypes are Morganella morganii and Morganella sibonii. These species inhabit in the intestinal tract of humans but more particularly in the urinary tract. M. morganii is predominantly associated with Urinary Tract Infections (UTI), sepsis and cutaneous disorders. The rate of infections caused by Morganella species is reported to be affected by difficulties in their isolation.[1] Morganella species-related bacteremia is occasionally found in hospitalized patients while being often reported in the general population. Sadly, the clinical features, virulence traits, and bacteremia brought on by M. morganii have scarcely been taken into account for treatment.[2]

Enterobacter, species, Citrobacter freundii, Serratia marcescens, and Morganella morganii are the major pathogens involved in hospital acquired infections. All of them are extensively reported in the infections of urinary tract, respiratory tract, cutaneous disorders, catheter associated infections and infections of the central nervous system (CNS). These microbes possess certain enzymes such as AmpC β-lactamases which enables them to confer resistance against extended spectrum cephalosporines. According to current literature, excessive productions of Extended Spectrum Beta Lactamase (ESBL) enzyme occur in these microbes. This factor can results in high population risk as fewer antibiotics are efficacious towards AmpC β-lactamase and ESBL producing microbes.[3]

First and second generation Cephalosporins, Aminopenicillins, and Augmentin are a few examples of antibiotics to which M. morganii might develop resistance due to the production of the

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AmpC lactamase enzyme. Mutations in M. morganii results in reduced production of AmpC enzyme conferring resistance in Morganella against Cephamycin, Monobactams and third generation Cephalosporines.[4] Tigecycline, Polymyxins, Nitrofurantoin and Tetracycline are the antimicrobial agents to which M. morganii exhibits resistance.[5] The development of more resistant genes, such as those which encode carbapenemase or resistance emerged against quinolones might limit the treatment strategies. Carbapenemase production in Enterobacteriaceae is a dominant issue affecting human health throughout the world. Carbapenemase are capable of degrading entire β lactam antimicrobial agents as well as carbapenems.[6]

There are multiple reasons of conducting this study. First of all, the importance of detecting Uropathogens as well as their antibiogram pattern will facilitate the consultants to recommend effective antibiotics. Second, due to the pervasive irrational use of antibiotics, resistance is increasing day by day, thus determining the antibiotic sensitivity of common selected pathogen is very much important. Finally, the frequency of ESBL and MBL among Morganella, Enterobacter, and E. coli species will provide a broader base for clinicians to effectively manage the cases against the said pathogens.

MATERIAL AND METHODS

The study project was a part of MS Microbiology thesis, so after the approval of the university research and ethics board, the data collection was carried out from 13-04-2019 to 17-07-2019 within three months until the required sample size of 200 met. Written informed consents were obtained from the participants and ensured the right of anonymity and confidentiality. Samples of urine were collected from adults of both genders, who came to the outdoor patients' departments with the suspected complaints of UTIs. Thus, samples were collected from those patients consecutively at the OPDs of Khyber Teaching Hospital (KTH) Peshawar. Only those patients were included who were not previously treated with medications during hospital visits.

The urine samples were collected in morning time in sterile container. The samples were inoculated on sterile CLED and MacConkey agar plates. Cysteine Lactose Electrolyte Deficient Agar was used as a differential media for culturing of urinary tract pathogens. Nutrient Agar was used for sub-culturing.[7,8]

Standard Gram staining procedure was used for microscopy of bacterial isolates. This Gram's staining procedure was followed for all bacterial isolates and were grouped as Gram negative and Gram positive, respectively. A 100X oil emulsion objective lens was used to examine the slides.

Isolated microorganisms were identified morphologically and biochemically by performing different tests. These included morphological, biochemical, and urease tests.

Through morphological tests, Morganella morganii was identified by the appearance of off-white and opaque colored colonies on CLED agar plates while the growth of Enterobacter and E.coli species was in the form of opaque yellow colonies on CLED agar plates.[9,10]

Five different types of biochemical tests i.e. Urease, Citrate, Triple Sugar Iron (TSI), Oxidase and Indole were executed for identifying Gram negative bacteria respectively.[10,11]

Urease test is used to isolate Salmonella, Morganella, Klebsiella and Yersinia species. Urease production turns the slop color from golden yellow to pink.

Use of a differential medium, such as TSI agar, allows for the identification and differentiation of Enterobacteriaceae pathogens. This test was used to identify those bacteria which release acid and gas due to fermentation of carbohydrate and also produce H_2S from cysteine and thiosulphate. The test organism was inoculated in TSI agar and then stored for 24 hours at 37^0C in an incubator.

Indole test is performed to identify ability of an organism to degrade tryptophan to indole. For differentiation Kovac's reagent was used. It helps to differentiate Enterobacteriaceae species by the formation of red or pink rings indicated positive result on the top.

A 0.5ml Kovac's reagent was mixed with 3ml one day old culture of peptone water which contained tryptophan. The production of red colored ring indicated the positive result. The bacteria which show indole positive results are P. vulgaris, V. cholera and M. morganii.

Light green to blue color change and turbidity indicates growth of bacteria in the test tube. This differentiates between the Enterobacteria and other bacteria.

The test organism was inoculated in the medium and subjected to incubation at 37°C for 24 hours. The medium was examined for blue color which is indication of positive test and no color change of the media is indication of the negative test.

The "Kirby Bauer disc diffusion" method was used to determine the susceptibility pattern as per CLSI guidelines.[12]

The filter paper discs with a proven antibiotic concentration were placed on Muller Hinton Agar (MHA) plates after inoculating MHA with pure colony of microorganism. Antibiotics placed on MHA inhibit bacterial growth in an area where drug concentration is sufficient to kill bacteria or prevent their spread. After formation of a clear visible zone, the inhibition zone of the test organism is calculated.

The Phenotypic detection of Extended Spectrum β Lactamase (ESBL) production in Enterobacteriaceae can be detected by performing a phenotypic confirmatory combined disk test i.e., "Double Disk Synergy Test" as recommended by CLSI.

The DDST is executed to detect those strains which are resistant to Cefotaxime such as the strains which excessively produce cephalosporinase and ESBL enzyme.

The Carbapenemase enzyme production in E. coli, Enterobacter and M. morganii is identified by Modified Hodge Test (MHT). The MHT is performed to detect for the Carbapenemase production, when tested isolate produces the enzyme and allows growth of a carbapenem resistant organism towards a carbapenem disc. The clover leaf like appearance of isolate indicates a positive MHT. A clover leaf like indentation of the tested organism was observed which indicated a positive MHT. Growth of the test organism was detected within the disc diffusion zone.

Analysis was done through Excel and descriptive statistics were presented through tables and graphs.

RESULTS

3.1 Demographics:

Of the 200 samples that were collected in total, 125 isolates were positive and demonstrated the growth of various Gram-negative bacteria. The remaining 75 isolates, however, were negative and did not exhibit any bacterial growth. 58 (46.4%) male patients and 67 (53.6%) female patients were identified in the positive isolates. The participants' average age was 38 $\pm$ 7 (SD).

3.2 Distribution of Bacterial Species among Positive Urine Samples:

The following groups of bacteria were recognized from the total samples analyzed based on Gram staining, colony morphology, general features, and biochemical assays. E. coli 52 (41.6%), Enterobacter 45 (36%) and M. morganii 28 (22.4%) were the three distinct bacterial species that were found.

3.3 Susceptibility Pattern of E. coli, Enterobacter and M. morganii:

Antibiotic sensitivity tests were performed on the positive bacterial isolates. Meropenem and Imipenem demonstrated the most effective antibacterial action. They both displayed the identical sensitivity of 85 (68.0%). The remaining antibiotics' sensitivity levels were: Amikacin 78 (62.4%), Piperacillin/Tazobactam 77 (61.6%), Gentamicin 63 (50.4%), Cefepime 54 (43.2%), Cefixime 52 (41.6%), Ceftazidime 49 (39.2%), Fosfomycin 48 (38.4%), and Co-trimoxazole 47 (37.6%), Ceftriaxone 46 (36.8%) showed in Table 01 & Graph 01).

3.4 Phenotypic Detection of Extended Spectrum β Lactamase Producing E. coli, Enterobacter and M. morganii:

According to the DDST results, out of 125 positive isolates, 76 (60.8%) were ESBL positive and 49 (39.2%) were ESBL negative. The bacterial species displayed resistance to ceftazidime, cefotaxime, and Augmentin. The proportions of ESBL positive and ESBL negative species in E. coli, Enterobacter, and M. morganii varied. As shown in table 2, there were 32 (25.6%) ESBL-positive E. coli isolates and 20 (16.0%) ESBL-negative isolates, 29 (23.2%) ESBL-positive isolates and 16 (12.8%) ESBL-negative isolates among Enterobacter species, and 15 (12.0%) ESBL-positive and 13 (10.4%) ESBL-negative isolates among M. morganii. as summarized in Table 02.

Table 01. Susceptibility Pattern of E. coli, Enterobacter and M. morganii (N=125)

Antibiotics	Sensitive	Resistant
Ampicillin	19 (15.2%)	106 (84.8%)
Piperacillin / Tazobactam	77 (61.6%)	48 (38.4%)
Ceftazidime	49 (39.2%)	76 (60.8%)
Ceftriaxone	46 (36.8%)	79 (63.2%)
Cefixime	52 (41.6%)	73 (58.4%)
Cefepime	54 (43.2%)	71 (56.8%)
Aztreonam	49 (39.2%)	76 (60.8%)
Imipenem	85 (68.0%)	40 (32.0%)
Meropenem	85 (68.0%)	40 (32.0%)
Gentamicin	63 (50.4%)	62 (49.6%)
Amikacin	78 (62.4%)	47 (37.6%)
Ofloxacin	79 (63.2%)	46 (36.8%)
Fosfomycin	48 (38.4%)	77 (61.6%)
Nitrofurantoin	41 (32.8%)	84 (67.2%)
Co-trimoxazole	47 (37.6%)	78 (62.4%)
Cefoparazone/Sulbactam	80 (64.0%)	45 (36.0%)

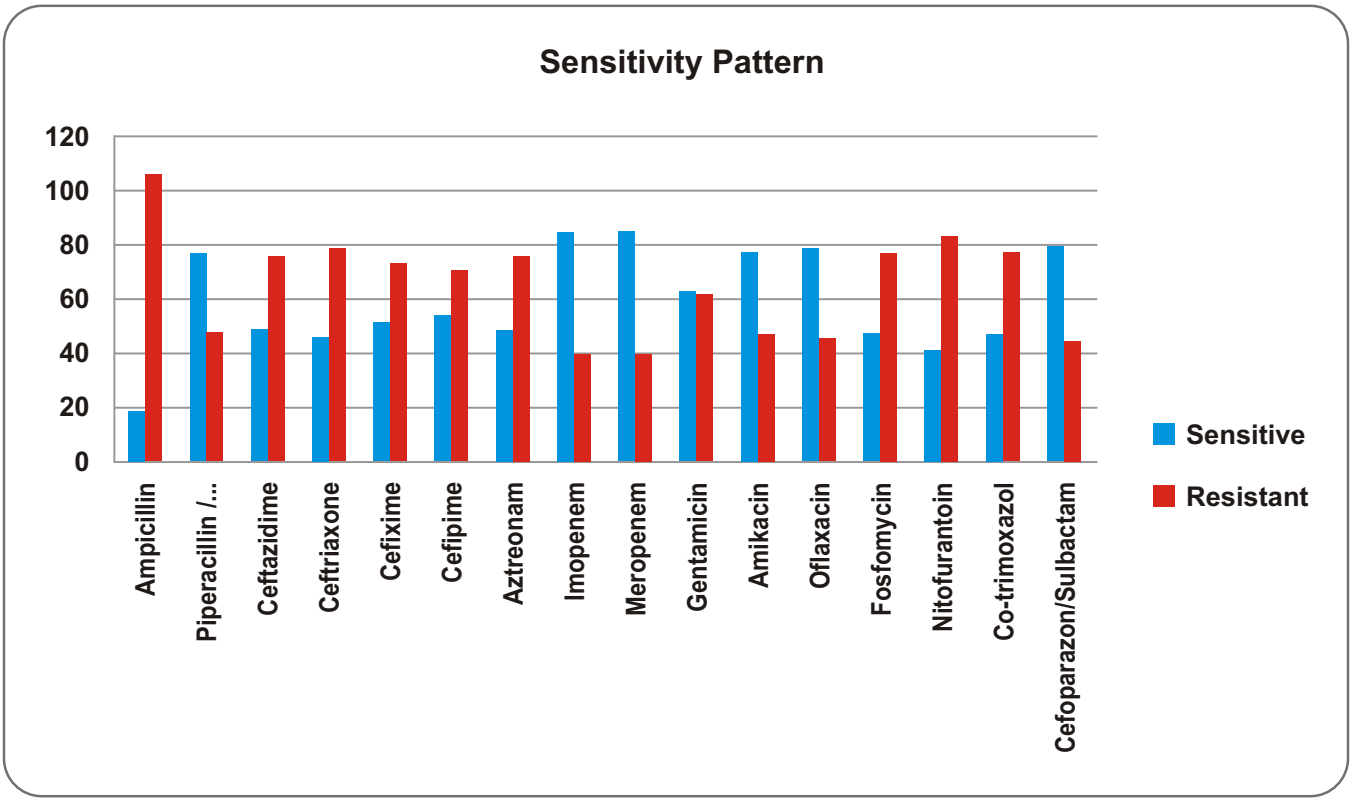


Figure 01. Antibiotics Susceptibility Pattern on Bar Graph (n=125)

Table 02. Phenotypic Detection of ESBL Positive and Negative E. coli, Enterobacter and M. morganii (n=125)

S. No.	Bacterial Species	ESBL Positive	ESBL Negative
1	E. coli	32 (25.6%)	20 (16.0%)
2	Enterobacter	29 (23.2%)	16 (12.8%)
3	Morganella	15 (12.0%)	13 (10.4%)
	Total	**76 (60.8%)**	**49 (39.2%)**

3.5 Phenotypic Detection of Metallo β Lactamase producing E. coli, Enterobacter and M. morganii:

MHT findings confirmed that 48 isolates (38.4%) were MHT negative and that 77 isolates (61.6%) were MHT positive. According to Table 03, M. morganii was represented by 19 (15.2%) MHT positive and 09 (7.2%) MHT negative E. coli species, Enterobacter was represented by 28 (22.4%) MHT positive and 17 (13.6%) MHT negative, and E. coli was represented by 30 (24.0%) MHT positive and 22 (17.6%) MHT negative.

Table 3. Phenotypic Detection of MHT Positive and Negative E. coli, Enterobacter and M. morganii (n=125)

S. No.	Bacterial Species	MHT Positive	MHT Negative
1	E. coli	30 (24.0%)	22 (17.6%)
2	Enterobacter	28 (22.4%)	17 (13.6%)
3	Morganella	19 (15.2%)	09 (7.2%)
	Total	**77 (61.6%)**	**48 (38.4%)**

DISCUSSION

This study was carried out to isolate and identify the E. coli, Enterobacter and M. morganii, in the urine samples and also to find antibiotic susceptibility and to detect ESBL/ MHT in the clinical isolates.

UTIs are mostly (about 95%) caused by single bacterial specie E. coli. Other bacteria that cause UTIs included Enterobacter, Proteus, Klebsiella, Pseudomonas Staphylococcus, Mycoplasma, Chlamydia, Serratia and Neisseria spp. It is estimated that approximately 35% of healthy women suffer from UTI symptoms and approximately 5% of women suffer from painful urination (dysuria) problems each year. The incidence of UTI in female is said to be greater than male in literature.[13]

In the current study, a total of 58 (46.4%) positive isolates were extracted from male while 67 (53.6%) urine specimens were positive among female patients. The following species were identified, E. coli 89 (50.8%), Enterobacter 45 (36%) and M. morganii 28 (22.4%). The microorganisms mostly prevalent among UTI patients were coagulase negative staphylococci (3.4%), Proteus mirabilis (5%), Klebsiella species (11.0%), Pseudomonas aeruginosa (7.5%), E. coli (46.9%), and Enterococcus species (12.8%) isolated in another such study.[14]

In this study, we found a high incidence of E. coli (50.8%). Our result for E.coli is in agreement with the Indian study which reported a high incidence of E.coli (50-90%).[15] The study of Choi et al. in Korea was a bit contrary to our findings, in which, the recorded frequency of ESBL positive microbes were Enterobacter species (12.8%), S. marcescens (12.4%), C. freundii (4.9%) and M. morganii (0%).[16] While in the current study, the frequency of ESBL positive microbes was 32 (42.1%) isolated E. coli, 29 (38.1%) isolated Enterobacter and 15 (19.7%) isolated Morganella spp. The frequency of antibiotic resistance among bacteria that cause UTIs in India was evaluated in a study that was published in 2021 in the Journal of Global Antimicrobial Resistance. With a prevalence of 66.7%, the study discovered that E. coli was the most prevalent bacterial species

isolated. High rates of resistance to routinely administered antibiotics like ampicillin, cefotaxime, and ciprofloxacin were also reported by the study. This study did not precisely assess ESBL production, in contrast to the preceding study. The urgent need for new UTI treatment options in India is highlighted by the high rates of resistance to widely administered antibiotics.[17] In a 2020 study, the frequency of ESBL-producing bacteria in patients with complex UTIs in China was investigated. With a prevalence of 68.3%, the study discovered that ESBL-producing E. coli was the most prevalent bacterial species isolated. High rates of resistance to routinely administered antibiotics like ceftriaxone, ciprofloxacin, and levofloxacin were also reported by the study. These results are consistent with the preceding investigation, which found significant rates of ESBL synthesis in E. coli isolates.[18] On the other hand, a 2019 study assessed the incidence of antibiotic resistance in UTI-causing bacteria in the United States. With a prevalence of 75.7%, the research showed that E. coli was the most prevalent bacterial species isolated. Additionally, the research reveals relatively low rates of resistance to antibiotics that are often prescribed, including trimethoprim-sulfamethoxazole, fosfomycin, and nitrofurantoin. In contrast to the research previously mentioned, which showed substantial rates of resistance to frequently administered antibiotics.[19,20]

According to our findings, Meropenem and Imipenem had the highest susceptibility rate of clinical bacterial isolates, at 68%, followed by Ofloxacin and Cefoparazone/Sulbactam (63.2%), Amikacin (62.4%), and Piperacillin/Tazobactam (61.6%), in that order. A Similar comprehensive Asia-Pacific study was presented with the findings that Meropenem was most sensitive antibiotic with susceptibility rate of 70%, followed by Piperacillin (65%), Augmentin (59%) and ciprofloxacin (47%).[20] Moreover, a couple of other studies reported very similar results where Meropenem showed highest activity followed by Ciprofloxacin, Ceftazidime, Cefoperazone and Nitrofurantoin showed low activity comparatively.[2122] The prevalence of UTI and the antibiotic resistance patterns of uropathogens were studied in research published between 2010 and 2020, according to a systematic review and meta-analysis in Iran, Escherichia coli (62%) were reported to be the most commonly isolated uropathogens in the review, which included studies from diverse nations. The analysis also discovered that ampicillin (86%), trimethoprim-sulfamethoxazole (64%), and ciprofloxacin (72%) had the highest rates of resistance. When choosing an antibiotic to treat a UTI, the review's authors advise practitioners to take local resistance tendencies into account.[23] In contrast, a more recent systematic review and meta-analysis carried out by Tufa et al. (2022) included research that reported the prevalence and antimicrobial resistance patterns of uropathogens in Africa that were published between 2015 and 2020. Escherichia coli (47.6%), Klebsiella pneumoniae (12.6%), and Enterococcus spp. (8.2%) were found to be the most commonly isolated uropathogens in the evaluation, which comprised studies from 22 African nations. The analysis also discovered that ampicillin (78.5%), trimethoprim-sulfamethoxazole (56.8%), and nalidixic acid (53.5%) had the highest rates of resistance. In order to determine the most effective empirical treatment for UTI, the authors of the review contend that ongoing monitoring of antimicrobial resistance patterns in Africa is necessary.[24] These findings from the comprehensive analysis provide more light on the frequency and patterns of uropathogen resistance, emphasizing the necessity for context-specific approaches to antibiotic choice for the treatment of UTIs.

The study adds a body of scientific knowledge which is clinically very significant and an impetus to the clinicians and policy makers that how unnecessarily prevailing practices of prescribing antibiotics and antibiotics resistance are developing among the UTIs' patients. The study has several limitations as well. The study only provides a snapshot or cross-section of the research problem. Furthermore, well-designed studies will further establish and warrant cause and effect. One centered data collection and relatively a small sample size with non-probability sampling techniques were limited the findings generalizability.

CONCLUSION

The study was successful in isolating and identifying E. coli, Enterobacter and M. morganii in urine samples, as well as in finding antibiotic susceptibility and detecting ESBL/ MHT in the clinical isolates. The study also revealed that Meropenem and Imipenem had the highest susceptibility rate of clinical bacterial isolates, at 68%, followed by Ofloxacin and Cefoparazon/ Sulbactam (63.2%), Amikacin (62.4%), and

Piperacillin/Tazobactam (61.6%), in that order. The findings of this study are clinically significant and an impetus to the clinicians and policy makers to consider the prevalence of antibiotic resistance in UTI patients. However, the study had some limitations such as small sample size and non-probability sampling techniques, which limited the generalizability of the findings. This study will also help the clinicians to consider the overuse of antibiotics without screening the causative organisms which will exacerbate the multidrug resistance issues.

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AUTHOR'S CONTRIBUTION
The following authors full fill authorship criteria as per ICMJE guidelines;

Shams H, Suliman M:	Substantial contribution to conception and design, drafting the manuscript, final approval, agreed to be accountable for all the work.
Noor U, Aziz A:	Substantial contribution to conception and design, data acquisition, drafting the manuscript, final approval, agreed to be accountable for all the work.
Khanum S, Naz N:	Analysis and interpretation of data, drafting the manuscript, final approval, agreed to be accountable for all the work.
Rehman S:	Analysis and interpretation of data, critical revision,final approval, agreed to be accountable for all the work.