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# Antimicrobial susceptibility patterns and CTX-M β-lactamase producing clinical isolates from burn patients in Islamabad, Pakistan

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ABSTRACT

**Objective:** To evaluate the prevalence of extended spectrum beta-lactamases (ESBL) in clinical isolates from burn patients using phenotypic and genotypic analyses.
**Methods:** During 2015–2016, a total of 126 samples were collected at a tertiary care hospital, Islamabad. Antibiotic sensitivity and ESBL prevalence were evaluated according to the Clinical Laboratory and Standards Institute, and molecular analysis of the CTX-M type ESBL gene was performed in 225 bacterial isolates from these samples.
**Results:** The most prevalent bacterial species were *Escherichia coli* (28.4%), *Pseudomonas aeruginosa* (22.2%), *Staphylococcus aureus* (19.6%), *Klebsiella pneumoniae* (16.4%), and coagulase-negative staphylococci (13.3%). Of the 225 bacterial isolates, 89 (39.5%) were found to be ESBL producers. The isolates were highly susceptible to meropenem (88%) and imipenem (84%), followed by the aminoglycoside amikacin (81%). Molecular epidemiology of the ESBL isolates indicated 19% prevalence of CTX-M. Resistance to antibiotics was exhibited by 28% isolates.
**Conclusions:** In the present study, bacteria such as *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, and *E. coli* isolated from burn patients exhibited resistance to one or more antibiotics and produced large amounts of ESBL. Further studies are needed to investigate the virulence and epidemiology of CTX-M type ESBL in clinical isolates from burn patients.

## 1. Introduction

In spite of continual advances in treatment of the burns, infection still remains a huge threat to patients.

Septic processes account for 73% of death within the initial five days of post-burn[1-5]. Furthermore, exudate around burn wound is highly nutritive for growth and proliferation of microorganism[1,6]. Skin is regarded as the first line defense against pathogens exposure; however, any thermal injury results in physical barrier disruption leading to pathogen invasion. Hence, skin damage along with compromised blood supply around wound and immunodeficiency increase acquisition of nosocomial infection in burn patients[1,7-12]. Moreover, long time hospitalization due to soil surrounding and hands of working staff also increases exposure of pathogens to patient's skin surface[1,13] resulting in high death rate in infected patients as compared to non-infected ones[14,15].

Although, availability of effective treatment using antibiotics has reduced mortality rate in burn patients[2], the persistent and improper use of broad spectrum antibiotics particularly the third generation cephalosporin leads to the development of multiple drug

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resistant strains thus limiting therapeutic options[3,13,16-19]. The primary cause for production of multiple drug resistant strains is β-lactamases particularly extended spectrum β-lactamases (ESBL) whose incidence is steadily increasing all over the world[9,16,19-24]. Most prevalent types of ESBL are TEM (TEM1-2), SHV-1 and CTX-M which are produced by Gram negative bacilli. The TEM and CTX-M type β-lactamases are produced by Enterobacteriaceae, while SHV type by *Klebsiella* spp. The global spreading of CTX-M ESBL has also been reported since last 10 years in different regions of Europe, and Asia including China and Pakistan, hence early detection of these multi-drug resistant bacteria is important in defining therapies for the prevention of nosocomial infections in the community[22]. In these strains *Escherichia coli* (*E. coli*) was observed to be pandemic[22].

Due to limited research work in Pakistan, the present study aimed at evaluating the bacteria from burn victims for antimicrobial susceptibility pattern, detection of ESBL production in clinical isolates and further molecular detection of CTX-M type ESBL gene responsible for prevalence of ESBL.

## 2. Materials and methods

### 2.1. Sample collection

This study was conducted at Department of Microbiology, Kohat University of Sciences and Technology, Pakistan and National Institute of Health (NIH), Islamabad, Pakistan from January 2015 to August 2016. A total of 126 wound samples were collected from burn care center of Islamabad under sterile conditions and cultured aerobically for 24 h at 37 ℃ in blood agar and McConkey agar for analyzing the morphological and biochemical characteristics.

### 2.2. Antibiotic susceptibility test

The antibiotic susceptibilities of isolated pathogens were checked through Kirby-Bauer's disk diffusion method under aseptic conditions. Briefly, sterile swabs were used to pick the inoculums and streaking was done over the entire sterile surface of Mueller-Hinton agar plate. The streaking was repeated 2–3 times to ensure the proper distribution of bacterial inoculums to obtain the even lawn. The antimicrobial disks of specific concentration were dispensed onto the medium surface gently and incubation was carried out for 24 h at 37 ℃. The antibiotics used were amikacin (Ak), ceftazidime (CAZ), ceftriaxone (CRO), piperacillin/tazobactam (TZP), amoxicillin-clavulanic acid (AMC), imipenem (IPM), meropenem (MEM), cephradine (CE), ciprofloxacin (CIP), cefaclor (CEC), cefpirome (CPO), cefoperazone + sulbactam (SCF), gentamicin (GTX), doxycycline (DO), ampicillin (AMP), and co-trimoxazole (SXT). The inhibition zone of bacterial growth around the antibiotic disc was measured in mm and results were interpreted as stated by Clinical Laboratory Standard Institute (CLSI) guidelines[25].

### 2.3. Detection of ESBL

Phenotypic detection of ESBLs was performed by proper inoculation with standardized inoculums (0.5 McFarland) on Mueller Hinton agar. The center of the Petri plate was amended with augmentin (20 μg amoxicillin and 10 μg clavulanic acid) disc and the third generation cephalosporins antibiotic discs of cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), and ceftriaxone (CRO, 30 μg) placed at 15 mm distance from augmentin. A difference of > 5 mm between the zone of inhibition of a single disc and combination with clavulanic acid indicates the presence of ESBL positive isolate.

### 2.4. DNA extraction and PCR detection of CTX-M gene

The whole genomic DNA was prepared from cultured strains. The *CTX-M* gene was amplified through specific oligonucleotides primers, forward primers CTX-M, F 5′-CGTCACGCTGTTGTTAGGAA-3′ and reverse primers CTX-M, R 3′-ACGGCTTTCTGCCTTAGGTT-5′. The total PCR mixture was 25 μL containing template DNA (1 μL), dNTPs mixture of 1.5 μL (0.2 mmol/L each), 2.5 μL 10× PCR buffer (*Ex Taq*), 0.5 μL *Taq* polymerase (1.25 IU) , 0.5 μL each primer stock solution (50 pmol/μL), 0.5 μL $MgCl_2$ and the remaining 18 μL volume was fulfilled by nuclease free water. Reaction conditions for primers were: initial denaturation for 5 min at 95 ℃, with 35 cycles of denaturation for 1 min at 95 ℃, annealing primer for 1 min at 58 ℃, and 2 min extension at 72 ℃ followed by a final extension for at least 5 min at 72 ℃. The 1.5% agarose gel was used to analyze the amplified products by comparing with standard molecular weight marker and subsequently amplified products were visualized by transilluminator using ethidium bromide.

## 3. Results

A total of 126 swabs were collected from burn patients attending the burn care center of Islamabad, Pakistan to identify isolates on suitable culture media by Gram staining and other biochemical tests. The isolated organisms were characterized for phenotypic production of ESBLs, *CTX-M* gene and antibiotic susceptibility. Out of 126 clinical samples, 90% (113/126) samples showed growth on selected media, while 10% (13/126) showed no growth. Out of 113 positive samples, 27% (31/113) samples had single isolates, while in 73% (82/113) samples, mixed microbial growth was found. The most prevalent bacterial species were *E. coli* (28.4%), *Pseudomonas aeruginosa* (*P. aeruginosa*) (22.2%), *Staphylococcus aureus* (*S. aureus*) (19.6%), *Klebsiella pneumoniae* (*K. pneumoniae*) (16.4%) and coagulase negative staphylococci (13.3%) (Tables 1 and 2).

**Table 1**
Distribution of bacterial isolates cultured from burn patients.

| No. | Bacterial isolates | No. of isolates | Percentage |
|---|---|---|---|
| 1 | *E. coli* | 64 | 28.4% |
| 2 | *P. aeruginosa* | 50 | 22.2% |
| 3 | *K. pneumoniae* | 37 | 16.5% |
| 4 | *S. aureus* | 44 | 19.6% |
| 5 | Coagulase negative staphylococci | 30 | 13.3% |

The most effective agents among the beta-lactams tested were meropenem (88.88%) and imipenem (84.88%). Furthermore, the isolates showed the lowest susceptibility to ampicillin (10.66%),

followed by cephradine (17.77%) and cefaclor (20%). The isolates showed intermediate susceptibility to cefpirome (46.66%), ceftriaxone (49.77%), and ceftazidime (62.66%). When combination of beta-lactams was tested, 70.66% isolates showed susceptibility to cefoperazone + sulbactam, 61.77% to piperacillin + tazobactam, while 26.66% to amoxicillin together with clavulanic acid. Further, among aminoglycosides, 81.77% isolates were susceptible to amikacin, and 32% to gentamicin. Among fluoroquinolones, maximum activity against isolates was displayed by ciprofloxacin (37.77%). Co-trimoxazole and doxycycline demonstrated susceptibility of 18.66% and 30.66% respectively (Table 3). A total of 63 (28%) isolates were resistant to more than 3 antibiotics. The widespread multi-drug resistant isolates also exhibited resistance to co-trimoxazole and doxycycline, and fluoroquinolones.

**Table 2**
Biochemical characteristics of bacterial isolates.

| Bacterial isolates | Coagulase | DNase | Motility | Indole | Citrate | Oxidase | Catalase |
|---|---|---|---|---|---|---|---|
| *E. coli* | - | - | + | + | - | - | - |
| *Klebsiella* | - | - | - | - | + | - | - |
| *Pseudomonas* | - | - | + | - | - | + | - |
| *Staphylococcus aureus* | + | + | - | - | - | - | + |

**Table 3**
Susceptibility of bacterial isolates to antimicrobial agents ($n$ = 225).

| Antibiotic | Resistant No. (%) | Intermediate No. (%) | Susceptible No. (%) |
|---|---|---|---|
| Ampicillin | 189 (84.00%) | 12 (5.33%) | 24 (10.66%) |
| Amoxicillin-clavulanate | 147 (65.33%) | 18 (8.00%) | 60 (26.66%) |
| Cephradine | 172 (76.44%) | 13 (5.77%) | 40 (17.77%) |
| Cefaclor | 169 (75.11%) | 11 (4.88%) | 45 (20.00%) |
| Ceftriaxone | 90 (40.00%) | 23 (10.22%) | 112 (49.77%) |
| Ceftazidime | 75 (33.33%) | 9 (4.00%) | 141 (62.66%) |
| Cefpirome | 50 (22.22%) | 6 (2.66%) | 105 (46.66%) |
| Cefoperazone + sulbactam | 39 (17.33%) | 27 (12.00%) | 159 (70.66%) |
| Piperacillin-tazobactam | 54 (24.00%) | 32 (14.22%) | 139 (61.77%) |
| Meropenem | 23 (10.22%) | 3 (1.33%) | 200 (88.88%) |
| Imipenem | 31 (13.77%) | 3 (1.33%) | 191(84.88%) |
| Gentamicin | 136 (60.44%) | 17 (7.55%) | 72 (32.00%) |
| Ciprofloxacin | 138 (61.33%) | 2 (0.88%) | 85 (37.77%) |
| Amikacin | 33 (14.66%) | 8 (3.55%) | 184 (81.77%) |
| Doxycycline | 145 (64.44%) | 11 (4.88%) | 69 (30.66%) |
| Co-trimoxazole | 176 (78.22%) | 7 (3.11%) | 42 (18.66%) |

In the present study, out of 225 isolates, 89 were found ESBL positive while 136 were ESBL negative. In 89 ESBL positive isolates, 59.55% showed susceptibility to aminoglycosides amikacin while 51.68% to combined antibiotics amoxillin-clavulanic acid. However, ESBL positive isolates were more resistant (28.08%) to amikacin (Table 4). Among all the antimicrobial agents tested, only ceftazidime and ceftriaxone showed less efficacy (12.35% and 15.73% susceptibility, respectively). Among fluoroquinolones levofloxacin also displayed better efficacy against ESBL isolates. In all 89 ESBL positive strains, susceptibility to levofloxacin was 47.19%. The susceptibility to imipenem was about three times higher in ESBL negative cases as compared to positive ones (61.02% *v.s* 23.59%). Moreover, 41 ESBL positive isolates were susceptible to piperacillin-tazobactam and ciprofloxacin. *CTX-M* gene analysis of ESBL positive isolates showed that only 19% (17/89) produced *CTX-M* gene. Furthermore, molecular characterization of CTX-M, class A β-lactamase, which is active against cefotaxime, was carried out by PCR and it was observed that $bla\text{-}_{\text{CTX-M}}$ was prevalent in 19% isolates (Figure 1).

**Table 4**
Antibiotic susceptibility patterns of ESBL positive and negative isolates ($n$ = 225).

| Antibiotic | ESBL positive ($n$ = 89) | | ESBL negative ($n$ = 136) | |
|---|---|---|---|---|
| | Resistant No. (%) | Sensitive No. (%) | Resistant No. (%) | Sensitive No. (%) |
| Ceftriaxone | 64 (71.91%) | 14 (15.73%) | 51 (37.50%) | 81 (59.55%) |
| Ceftazidime | 67 (75.28%) | 11 (12.35%) | 57 (41.91%) | 75 (55.14%) |
| Amikacin | 25 (28.08%) | 53(59.55%) | 41 (30.14%) | 91 (66.91%) |
| Ciprofloxacin | 30 (33.70%) | 41 (46.06%) | 41 (30.14%) | 69 (50.73%) |
| Amoxacillin-clavulanic acid | 32 (35.95%) | 46 (51.68%) | 42 (30.88%) | 92 (67.64%) |
| Levofloxacin | 36 (40.44%) | 42 (47.19%) | 49 (36.02%) | 77 (56.16%) |
| Tobramycin | 57 (64.04%) | 21 (23.59%) | 52 (38.23%) | 72 (52.94%) |
| Imipenem | 54 (60.67%) | 21 (23.59%) | 35 (25.73%) | 83 (61.02%) |
| Piperacillin-tazobactam | 31 (34.83%) | 41 (46.06%) | 43(31.61%) | 77 (56.61%) |

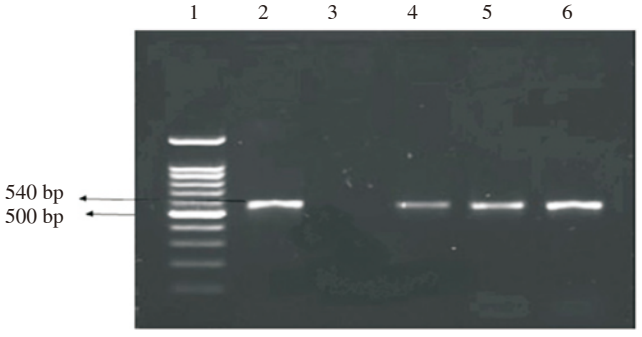


**Figure 1.** Result of the amplified product on 1.5% gel.
Lane 1: Ladder (100 kb); Lane 2: Positive control; Lane 3: Negative control; Lanes 4–6: Positive CTX-M sample showing band size around 540 bp.

## 4. Discussion

Burn wound infections are globally considered as life threatening due to high morbidity and mortality. Further, multi-drug resistance due to long time hospitalization, contaminated environment and high ESBL production rate by Enterobacteriaceae is challenging for treatment, thus identification of microorganism from burn wounds and their local susceptibility patterns can provide guidance for empiric antimicrobial therapy. In the present study, presence of multiple isolates from 126 samples was observed.

Gram negative organisms have been recognized as dominant pathogen in burn patients[18,26-28] and among these, the most frequent species were *Klebsiella*, *E. coli*, *Pseudomonas* and *Proteus*[1,4,10]. However, other Gram negative isolates like *Acinetobacter* may also be found in burn wounds[1,5,29]. In the present study, *E. coli* (28.4%) was the most prevalent bacterial species as similar to other reports[13] followed by *P. aeruginosa* (22.2%), *S. aureus* (19.6%), *K. pneumoniae* (16.4%), and coagulase-negative staphylococci (13.3%) (Tables 1 and 2). However, *P. aeruginosa* as the most

prevalent species (37%) followed by *K. pneumoniae* (15%) and *S. aureus* (12%) has been reported in other studies[1,5]; the prevalence of *P. aeruginosa* in the present study was comparable to that in the previous report[5]. Moreover, *S. aureus* (25%) followed by *P. aeruginosa* (13%) and coagulase-negative staphylococci (9%) were found in another report[7]. This may be due to continuous altering pattern of bacterial isolates in burn wound.

The antibiotic susceptibility patterns revealed that the most potent antimicrobial agents were meropenem and imipenem with 88.88% and 84.88% susceptibility which resembles to the findings of previous literature[19,30]. Similarly, high activity of aminoglycosides has been investigated[19,31] and in the current study amikacin showed high susceptibility (81.77%) against the isolates. While the data revealed that the isolates showed high resistance to ampicillin (84%), and in the previous studies conducted in Pakistan, isolates were highly resistant to penicillin[19,32,33]. Moreover, 39.5% (89/225) isolates were ESBL positive while remaining 60.5% (136/225) were ESBL negative and high susceptibility was observed in ESBL negative isolates (Table 4), which is similar to the finding of other works[19,32].

The persistent use of antibiotics due to increased selection pressure in hospitals often leads to MDR microorganisms and we found that 28% isolates were resistant to three or more antibiotics, which was practically the same as that previously reported[34]. However, higher percentage of MDR microorganisms (upto 73%) has also been reported[34]. Moreover, the period of hospitalization, hygienic conditions and drinking water quality also propagate MDR microbes as it has been reported that short-time stay of burn patients in hospital (7 days) led to 10% colonization of microbes while long-time stay (50 days) increased colonization up to 90%[35]. Another study reported that poor hygienic conditions such as contaminated drinking water promote Enterobacteriaceae strains circulation leading to MDR[21].

In routine laboratory tests, ESBLs are usually detected phenotypically; however, genotypic confirmation is essential for epidemiological studies in hospitals, and in the present study *CTX-M* gene was predominantly found in *E. coli* and *Klebsiella* spp. Similar finding has already been reported that all strains of *E. coli* had *CTX-M* gene[21]. It has also been documented that clinical isolates of *E. coli* and *Klebsiella* spp. are resistant to many antibiotics such as aminoglycosides, cotrimoxazole and ciprofloxacin, but none of the resistance was displayed to carbapenem[21,36], which was generated due to CTX-M enzyme production. Piperacillin-tazobactam antibiotics combination exhibited even more susceptibility against CTX-M-producing isolates. The isolate in the UK produces an OXA-1 β-lactamase, resulting in resistance to multiple or combinations of antibiotics[34].

Furthermore, molecular characterization of CTX-M, class A β-lactamase, which is active against cefotaxime, was carried out by PCR and it was observed that *bla*-$_{CTX-M}$ was prevalent in 19% isolates (Figure 1). However, this prevalence was comparatively higher than that in previous report (19% *v.s* 2.3%)[18]. We believed that, the emergence of *CTX-M* gene conferred antibiotic resistance in microbes. The CTX-M emergence from Pakistan is frightening; therefore further studies are required for molecular epidemiology of CTX-M type ESBLs.

The increasing resistance to antibiotics among burn isolates is a matter of concern, as there is limited treatment against multi drug resistance. The *CTX-M* gene is a new emerging gene for beta lactamase resistance from burn patients in Pakistan. In Pakistan, there is a great need for genotypic characterization of responsible gene. Furthermore, considering variety of burn isolates, regular microbiological surveillance and their antimicrobial resistance pattern will help us in properly formulating antibiotic therapy and reducing mortality from septic infections.

## Conflict of interest statement

We declare that we have no conflict of interest.

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