Emergence of $bla_{\text{TEM}}$, $bla_{\text{CTX-M}}$, $bla_{\text{SHV}}$ and $bla_{\text{OXA}}$ genes in multidrug-resistant Enterobacteriaceae and Acinetobacter baumannii in Saudi Arabia

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Abstract. Multidrug-resistant (MDR) patterns due to extended-spectrum $\beta$-lactamase (ESBL) production in pathogenic bacteria are now becoming prevalent in hospitals worldwide, posing a public health challenge. The aim of the present study was to determine the antibiotic susceptibility patterns and distribution of the $bla_{\text{TEM}}$, $bla_{\text{CTX-M}}$, $bla_{\text{SHV}}$ and $bla_{\text{OXA}}$ ESBL resistance genes in MDR Enterobacteriaceae and Acinetobacter baumannii (A. baumannii). A cross-sectional study was conducted between September 2017 and August 2018 in the King Abdullah Hospital (Bisha, Saudi Arabia). Bacterial isolates were collected from the clinical samples of patients; these were identified and screened for ESBL production and their antibiotic susceptibility was examined using standard microbiology methods. Multiplex-PCR runs were performed to identify genes encoding ESBL producers. DNA sequencing analysis was used to identify the specific gene variants. Of the 274 isolates, 173 (63.1%) exhibited MDR patterns to different antibiotics. A. baumannii revealed the highest resistance rates for cefuroxime (100%), gentamicin (88%) and amikacin (86%). Klebsiella pneumoniae (K. pneumoniae) isolates had the highest resistance rates for cefuroxime (98%), aztreonam and trimethoprim/sulfamethoxazole (87% for each). Escherichia coli (E. coli) exhibited high resistance rates for trimethoprim/sulfamethoxazole (92%) and cefuroxime (87%). Of the 173 MDR isolates, 78 (45.1%) exhibited ESBL production. Of these, 88.9% (72/78) carried ESBL genes. The most prevalent gene-encoding isolates were $bla_{\text{TEM}}$ (84.7%), followed by $bla_{\text{CTX-M}}$ (33.3%), $bla_{\text{SHV}}$ (27%) and $bla_{\text{OXA}}$ (1.4%). A single $bla_{\text{TEM}}$ gene was predominantly produced by K. pneumoniae (60.7%), A. baumannii (78.9%) and Proteus mirabilis (80%), whereas $bla_{\text{CTX-M}}$ was harbored by E. coli (33.3%). The co-existence of two different genes in a single bacterium was revealed in 22.2% of isolates, commonly between $bla_{\text{TEM}}$ and $bla_{\text{CTX-M}}$ (19.4%). Sequencing analysis revealed that $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-1}}$ were predominant variants of the $bla_{\text{CTX-M}}$ and $bla_{\text{TEM}}$ genes, respectively. The present study revealed a diversity of ESBL genes in Gram-negative bacterial isolates, with $bla_{\text{TEM}}$ being the most prevalent type. The emergence of various ESBL genes with several co-existing genotypes is alarming, rendering extensive surveillance studies necessary to understand the transmission and epidemiology of such resistant gene-carrying isolates.

Introduction

In recent years, the production of extended-spectrum $\beta$-lactamases (ESBL) has become the main mechanism of resistance to $\beta$-lactam and other antibiotics in Enterobacteriaceae and Acinetobacter baumannii (A. baumannii) (1). ESBL enzymes confer resistance to penicillins, cephalosporins, monobactams and other antibiotic classes (2). Multidrug-resistant (MDR) patterns due to ESBL production in pathogenic bacteria are now becoming prevalent in hospitals worldwide, posing a public health challenge, including treatment failure, prolonged hospital stay and increased mortality rates (3-5).

Recently, >300 different ESBL types have been described in Gram-negative bacteria (6,7). The $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ types have been recognized as the most prevalent ESBL genes conferring antibiotic resistance in pathogenic bacteria worldwide (8-10). Previous studies have revealed that the number of clinical isolates harboring the $bla_{\text{CTX-M}}$ gene type has also increased in the last few years (2,11). The $bla_{\text{CTX-M}}$ family includes >130 $\beta$-lactamase variants classified into five distinct groups: $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M-25}}$ (12).

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The genetic diversity of ESBL-producing Enterobacteriaceae and A. baumannii has progressively increased, posing challenges to hospital authorities due to their ability to confer antibiotic susceptibility and limit therapeutic options (13,14). The characterization of resistance genes encoding ESBL-producing microorganisms is a powerful tool for developing evidence-based guidelines for combating antibiotic resistance in the clinical setting (11).

In Saudi Arabia, Gram-negative bacteria-harboring ESBL resistance genes have been studied, with most studies emerging from the central and eastern regions (5,15-19). Despite the spread of antibiotic resistance among bacterial pathogens in the southern region, data on the distribution of ESBL resistance continue to be sparse (20). A previous study performed at the Aseer Central Hospital, a regional hospital in the southern region, identified MDR patterns among 98.1% of Acinetobacter species recovered from patients at intensive care units (22). In addition, another study conducted in the same hospital identified a high distribution of class D carbapenemase-encoding genes in A. baumannii, mainly ISAba1/OXA-23 and ISAba1/OXA-24 carbapenemases, which is alarming and presents an emerging public health threat (23). The emergence of MDR A. baumannii bacteremia in the southern region of Saudi Arabia has been well documented as an important health problem (22,24,25). A recent study reported the high frequency of MDR Gram-negative bacteria and a rate of ESBL production of 27% in patients at the King Abdullah Hospital, a referral hospital in Bisha, in the southern region of the country (20).

Due to the lack of information on the genotyping of ESBL producers and their MDR patterns in southern Saudi Arabia, the present study aimed to determine the antibiotic susceptibility patterns and distribution of ESBL genes in Enterobacteriaceae and A. baumannii isolates collected from clinical specimens of patients. The findings of the present study facilitated the implementation of infection control measures and provided epidemiological data to prevent spreading of MDR bacteria. The data also provided guidelines for the use of antibiotics in the clinical settings and improved the management of patients suffering from infections caused by Enterobacteriaceae and A. baumannii.

Materials and methods

Study design and setting. A cross-sectional study was conducted between September 2017 and August 2018 at the King Abdullah Hospital (Bisha, Saudi Arabia). A total of 274 Enterobacteriaceae and A. baumannii were recovered from patients as a part of the treatment and diagnosis procedure for infections. The clinical samples were collected from the patients as a part of routine investigations of infectious agents in the hospital microbiology laboratory. Therefore, consent letters were not obtained from the patients as per the study nature. Various clinical samples of urine (n=96), sputum (n=84), wound swab (n=51), blood (n=27), high vaginal swab (n=5), tracheal aspirate (n=5), umbilical discharge (n=3), cerebrospinal fluid (n=2) and eye swab (n=1) collected from 274 patients suffering from different bacterial infections were included in the study. Discharge from umbilical stump was collected from neonatal and/or infant patients in the wards using sterile swab soaked with normal saline and submitted to the laboratory. High vaginal swab was taken from female patients by clinicians and sent for microbiological examinations. Clinical specimens with incomplete personal information of the patients were excluded from the study. The average age of the patients was 46.0±25.5 years, including 158 females and 116 males. In total, 32 patients were aged 2-17 years, 97 were aged 18-40 years, 57 were aged 41-65 years and 88 were aged >65 years. Patients of <2 years old were excluded from the study. The hospital is a referral hospital in the southern region (365 beds) with different specialties serving the Bisha province and the surrounding areas. The Research and Ethics committee at the College of Medicine, University of Bisha (Bisha, Saudi Arabia) reviewed and approved the present study protocol (approval no. UBCOM/1438-05/04).

Isolation and identification of bacteria. Enterobacteriaceae and A. baumannii were collected from the microbiology laboratory of King Abdullah Hospital during the routine processing of the clinical specimens of patients. Preliminary isolation and identification of bacteria were based on conventional microbiological methods. Briefly, Isolation of bacterial pathogens from specimens of urine, stool, sputum and other body fluids were performed by inoculating one loopful of each sample onto MacConkey agar plates (Oxoid, Ltd.) using a sterile disposable plastic loop (10-µl loop). Specimens of wounds, eye, umbilical and vaginal swabs were inoculated directly onto MacConkey agar plates by streaking them onto a small area of the plate. A disposable sterile plastic loop (1-µl loop) was used for cross-streaking to spread the inoculum over the surface of each plate to obtain single colonies of the suspected bacterial pathogen. Specimen of blood (5 ml) were extracted under aseptic conditions, transferred immediately into sterile bottles containing brain heart infusion broth (Oxoid, Ltd.), incubated at 37°C with 5% CO₂, and examined daily for turbidity for ≤7 days. If turbidity was observed, a 10-µl loopful of the blood sample was subcultured onto MacConkey agar plates for isolation of the suspected Gram-negative pathogen after aerobic incubation at 37°C for 24 h. The isolate is tentatively identified based on the colony morphology, gram staining and oxidase test and the API 20 E Gram-Negative Microbial Identification Kit (cat. no. 20160; bioMerieux SA). Then, full identification of bacterial isolates was confirmed using the Phoenix system identification assay (Becton, Dickinson and Company). One single bacterial isolate from the clinical sample of each patient was included in the present study.

Screening of ESBL production. Phenotypic ESBL production among isolates was examined using a double-disc synergy test (DDST) as previously described (26) and the decreased susceptibility to cefuroxime, ceftazidime and cefotaxime was examined according to the Clinical and Laboratory Standard Institute (CLSI) recommendations (27). Bacterial isolates yielded positive results with DDST, when subjected to a multiplex-PCR amplification assay to detect blaTEM, blaSHV, blaCTX-M and blaOXA-1 resistance genes.

Antibiotic susceptibility testing of ESBL-producing bacteria. Antibiotic susceptibility testing of the Enterobacteriaceae
and *A. baumannii* was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar medium (Oxoid) as per the CLSI guidelines (27). The following antibiotics with known concentrations recommended by the CLSI were examined: Amikacin (30 µg), amoxicillin/clavulanate (20/10 µg), aztreonam (30 µg), cefepime (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), colistin (10 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), nitrofurantoin (50 µg), piperacillin (100 µg), piperacillin/tazobactam (100/10 µg), tobramycin (10 µg) and trimethoprim/sulfamethoxazole (23.75 µg/1.25 µg; Oxoid). *E. coli* American Type Culture Collection (ATCC) 25922 served as a control strain for antibiotic susceptibility examination. The final antibiotic susceptibility results of bacterial pathogens were interpreted using the 2017 CLSI breakpoints to categorize the isolates as susceptible or resistant. All intermediate results were considered resistant strains. ‘A susceptible category indicates that the isolates of the patient respond to the usually achievable concentrations of that antibiotic when the dosage is recommended to treat that type of infection and bacterial species. Conversely, the resistant category indicates that the isolates of the patient are not inhibited by the usually achievable concentrations of that antibiotic with the dosages usually used with that drug’ (27). Isolates were defined as MDR when they were resistant to at least three antibiotics from different classes.

**Multiplex-PCR for the detection of ESBL genes.** Multiplex-PCR runs were performed to identify the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub> and *bla*<sub>OXA-1</sub> genes. A pair of six specific oligonucleotide primers (Eurofins Scientific) were used in the PCR reaction, as previously described (28). DNA was extracted from ESBL-producing isolates using the boiling method as previously described (29). The amplification of ESBL genes was then carried out in a total reaction volume of 50 µl. Each reaction mixture contained 25 µl HotStarTaq Plus Master Mix (cat. no. 203643; Qiagen GmbH), 4 µl DNA template, a variable volume of a specific primer group (Table I) and 9 µl nuclease-free water. The Eppendorf Mastercycler Gradient instrument (Eppendorf) was used for the amplification of target genes with the following optimal cycling conditions: Initial heat activation at 95˚C for 5 min, 35 cycles of denaturation at 94˚C for 45 sec, annealing at 53˚C for 45 sec, extension at 72˚C for 1 min and a final extension at 72˚C for 10 min. The amplification products were visualized under ultraviolet illumination at a wavelength of 312 nm, after running at 85 volts for 60 min on 2% agarose containing ethidium bromide (1 µg/ml). A 100-bp DNA ladder (cat. no. 239045; Qiagen GmbH) was used as a standard molecular weight to determine the size of PCR products. DNA from reference *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA-like</sub>-positive strains was used as a positive control. **DNA sequencing.** Random PCR products of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub> and *bla*<sub>OXA-like</sub>-positive samples were selected for DNA sequencing to identify the specific gene subtypes. A total of ~30 µl PCR products were sealed in sterile Eppendorf tubes and sent to Macrogen, Inc. for sequencing. PCR products were sequenced on an ABI PRISM® 3730XL Analyzer (96 capillary types) using the same primer sets (Table I). The results were obtained from the website of the company.

### Table I. The frequency of extended-spectrum β-lactamase resistance genes among Gram-negative bacteria (n=72) as detected by multiplex PCR using different primers.

| Resistance gene | N (%) | Sequence | Length (base pairs) | Band size (base pairs) |
|-----------------|-------|----------|---------------------|-----------------------|
| *bla*<sub>TEM</sub> | 61 (84.7) | F: 5'-CATTTCCGTGTCGCCCTTATTC-3' | 22 | 800 |
| *bla*<sub>CTX-M</sub> | 24 (33.3) | R: 5'-CGTTCATCCATAGTTGCCTGAC-3' | 22 |
| *bla*<sub>CTX-M</sub> group 1 | 20 (27.2) | F: 5'-TTAGGAARTGTGGCCGTGYA-3' | 20 | 688 |
| *bla*<sub>CTX-M</sub> group 2 | 3 (4.2) | R: 5'-CGATATCGTTGGTGGTRCCAT-3' | 21 |
| *bla*<sub>CTX-M</sub> group 9 | 3 (4.2) | F: 5'-TCAAGGCTTCGCCAAGTGTGT-3' | 19 | 561 |
| *bla*<sub>CTX-M</sub> group 8/25 | 0.0 | R: 5'-TACAGCCCGGGAAGTGTAYAT-3' | 19 |
| *bla*<sub>SHV</sub> | 2 (2.7) | F: 5'-AGCCGGCTTTGAGCAAATAC-3' | 21 | 713 |
| *bla*<sub>OXA-1</sub> | 1 (1.4) | R: 5'-ATCCCGAGATAATCACCAC-3' | 21 |

F, forward; R, reverse.
 Similarities in the nucleotide sequences were compared on the GenBank database of the National Center for Biotechnology Information website using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences obtained for \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{CTX-M}} \) and \( \text{bla}_{\text{SHV}} \) genes have been deposited in the GenBank database (http://getentry.ddbj.nig.ac.jp/) under accession numbers LC636038 to LC636063.

**Statistical analysis.** Statistical analysis was performed using SPSS 22.0 (IBM Corp.). Simple descriptive statistics were used to calculate antibiotic-resistant patterns, and the prevalence and distribution of ESBL resistance genes. Outcome data were stated as proportions in frequencies and means ± standard deviation.

**Results**

**Bacterial isolates.** A total of 274 Enterobacteriaceae and A. baumannii isolates obtained from the clinical samples of patients were used in the present study. The isolates were recovered from samples of urine, sputum, wound swab, blood, high vaginal swab, tracheal aspirate, umbilical discharge, eye swab and cerebral spinal fluid. This indicated that Enterobacteriaceae and A. baumannii could cause infections throughout body systems and sites, including genito-urinary, respiratory, bloodstream, central nervous and soft tissue infections, which was consistent with a recent study (30).

**Resistance patterns of Enterobacteriaceae and A. baumannii.** Out of the 274 isolates, 63.1% (n=173) exhibited MDR patterns to different classes of antibiotics, and the prevalence and distribution of ESBL resistance genes. Outcome data were stated as proportions in frequencies and means ± standard deviation.

**Distribution of ESBL genes among MDR isolates.** Of the 173 MDR Enterobacteriaceae and A. baumannii isolates, 78 (45.1%) exhibited ESBL production. Of the 78 isolates, 72 (92.3%) carried ESBL genes as determined using multiplex-PCR. Fig. 2 presents an example of multiplex-PCR results revealed during the present study. Of the 78 isolates, 53 were Enterobacteriaceae (K. pneumoniae, 28; E. coli, 18; P. mirabilis, 5 and Enterobacter cloacae, 2) and 19 were A. baumannii (Fig. 3). Collectively, the most prevalent ESBL resistance genes in the isolates were \( \text{bla}_{\text{TEM}} \) (84.7%) followed by \( \text{bla}_{\text{CTX-M}} \) (33.3%), \( \text{bla}_{\text{SHV}} \) (2.7%) and \( \text{bla}_{\text{OXA-1}} \) (1.4%). The most frequent \( \text{bla}_{\text{CTX-M}} \) group was the \( \text{bla}_{\text{CTX-M\text{-}1}} \) group (27.2%), followed by the \( \text{bla}_{\text{CTX-M\text{-}9}} \) group 2 and 9 (4.2%), whereas none of the isolates carried the \( \text{bla}_{\text{CTX-M\text{-}8/25}} \) group (77%).

**Frequency of ESBL genes in Enterobacteriaceae and A. baumannii.** Fig. 3 illustrates the distribution of ESBL genes
| Agent               | A. baumannii (n=49) | K. pneumonia (n=54) | E. coli (n=38) | Proteus mirabilis (n=17) | Enterobacter cloacae (n=6) | Citrobacter freundii (n=1) | Providencia rettgeri (n=2) | Proteus vulgaris (n=2) | Morganella morganii (n=2) | K. oxytoca (n=2) |
|---------------------|---------------------|---------------------|----------------|--------------------------|---------------------------|--------------------------|--------------------------|--------------------------|--------------------------|-------------------|
| Amikacin           | 86 (42)             | 41 (22)             | 16 (6)         | 35 (6)                   | 33 (2)                    | 0.0 (0)                   | 50 (1)                   | 50 (1)                   | 50 (1)                   | 50 (1)             |
| Amoxicillin/clavulanate | 92 (45)         | 69 (37)             | 37 (14)        | 88 (15)                  | 50 (3)                    | 100 (1)                  | 50 (1)                   | 50 (1)                   | 50 (1)                   | 50 (1)             |
| Aztreonam          | 96 (47)             | 87 (47)             | 68 (26)        | 65 (11)                  | 83 (5)                    | 100 (1)                  | 50 (1)                   | 50 (1)                   | 100 (2)                  | 100 (2)            |
| Cefepime           | 94 (46)             | 82 (44)             | 66 (25)        | 82 (14)                  | 50 (3)                    | 0.0 (0)                   | 50 (1)                   | 0.0 (0)                   | 50 (1)                   | 0.0 (0)             |
| Cefotaxime         | 96 (47)             | 83 (45)             | 66 (25)        | 88 (15)                  | 33 (2)                    | 100 (1)                  | 50 (1)                   | 50 (1)                   | 50 (1)                   | 50 (1)             |
| Ceftazidime        | 94 (46)             | 80 (43)             | 71 (27)        | 59 (10)                  | 50 (3)                    | 0.0 (0)                   | 0.0 (0)                   | 50 (1)                   | 50 (1)                   | 0.0 (0)             |
| Cefuroxime         | 100 (49)            | 98 (53)             | 87 (33)        | 88 (15)                  | 83 (5)                    | 100 (1)                  | 100 (2)                  | 100 (2)                  | 100 (2)                  | 50 (1)             |
| Ciprofloxacin      | 96 (47)             | 61 (33)             | 58 (22)        | 82 (14)                  | 33 (2)                    | 100 (1)                  | 100 (2)                  | 0.0 (0)                   | 50 (1)                   | 100 (2)            |
| Colistin           | 6 (3)               | 4.0 (2)             | 3.0 (1)        | 6.0 (1)                  | 0.0 (0)                   | 0.0 (0)                   | 0.0 (0)                   | 0.0 (0)                   | 100 (2)                  | 0.0 (0)             |
| Foxitin            | 92 (45)             | 46 (25)             | 18 (7)         | 59 (10)                  | 50 (3)                    | 100 (1)                  | 0.0 (0)                   | 0.0 (0)                   | 100 (2)                  | 50 (1)             |
| Gentamicin         | 88 (43)             | 61 (33)             | 37 (14)        | 53 (9)                   | 17 (1)                    | 0.0 (0)                   | 50 (1)                   | 50 (1)                   | 100 (2)                  | 0.0 (0)             |
| Imipenem           | 94 (46)             | 35 (19)             | 13 (5)         | 29 (5)                   | 17 (1)                    | 0.0 (0)                   | 50 (1)                   | 50 (1)                   | 100 (2)                  | 0.0 (0)             |
| Meropenem          | 92 (45)             | 33 (18)             | 13 (5)         | 24 (4)                   | 0.0 (0)                   | 0.0 (0)                   | 0.0 (0)                   | 0.0 (0)                   | 100 (2)                  | 50 (1)             |
| Nitrofurantoin     | 96 (47)             | 69 (37)             | 37 (14)        | 71 (12)                  | 67 (4)                    | 0.0 (0)                   | 100 (2)                  | 0.0 (0)                   | 50 (1)                   | 50 (1)             |
| Ofloxacin          | 94 (46)             | 63 (34)             | 50 (19)        | 77 (13)                  | 33 (2)                    | 100 (1)                  | 100 (2)                  | 0.0 (0)                   | 50 (1)                   | 100 (2)            |
| Piperacillin       | 94 (46)             | 61 (33)             | 47 (18)        | 65 (11)                  | 50 (3)                    | 0.0 (0)                   | 50 (1)                   | 50 (1)                   | 100 (2)                  | 50 (1)             |
| Piperacillin/ tazobactam | 94 (46)         | 54 (29)             | 40 (15)        | 35 (6)                   | 50 (3)                    | 0.0 (0)                   | 50 (1)                   | 50 (1)                   | 100 (2)                  | 50 (1)             |
| Tobramycin         | 90 (44)             | 59 (32)             | 37 (14)        | 53 (9)                   | 33 (2)                    | 0.0 (0)                   | 50 (1)                   | 50 (1)                   | 50 (1)                   | 50 (1)             |
| Trimethoprim/ sulfamethoxazole | 96 (47)         | 87 (47)             | 92 (35)        | 100 (17)                 | 100 (6)                  | 100 (1)                  | 100 (2)                  | 50 (1)                   | 50 (1)                   | 100 (2)            |
among Gram-negative bacterial isolates. A single \textit{bla}_{\text{TEM}} gene was predominantly produced by \textit{K. pneumoniae} (60.7%), \textit{A. baumannii} (78.9%) and \textit{P. mirabilis} (80%), while \textit{bla}_{\text{CTX-M}} was commonly produced by \textit{E. coli} (33.3%).

Out of the total PCR-positive isolates (n=72), the co-existence of two different genes in a single isolate was revealed in 16 (22.2%) strains. This combination was mainly observed between \textit{bla}_{\text{TEM}} and \textit{bla}_{\text{CTX-M}} (19.4%; 14/72) and between \textit{bla}_{\text{TEM}} and \textit{bla}_{\text{SHV}} (2.8%, 2/72) genes. Among the isolates carrying a combination of \textit{bla}_{\text{TEM}} and \textit{bla}_{\text{CTX-M}} genes (n=14), the majority were \textit{K. pneumoniae} (50%, n=7) and \textit{E. coli} (28.6%, n=4; Fig. 3).

\textbf{Distribution of ESBL genes according to isolate sources.} The distribution of various ESBL resistance genes among the clinical samples of patients is revealed in Table III. Bacterial isolates encoding various ESBL resistance genes were commonly recovered from sputum (n=30), followed by urine (n=18), wound (n=13) and blood (n=8) specimens. A single \textit{bla}_{\text{TEM}} gene was frequently detected among isolates from sputum (73.3%; 22/30), urine (61.1%; 11/18) and wound (53.8%; 7/13) specimens. The highest frequency of a single \textit{bla}_{\text{CTX-M}} gene was detected among wound isolates (23.1%; 3/13) compared with sputum (10%), blood (12.5%; 1/8) and urine (11.1%; 2/18) isolates. The co-existence of \textit{bla}_{\text{TEM}} and \textit{bla}_{\text{CTX-M}} genes was observed in 37.5% (3/8) of blood isolates, 23.1% (3/13) of wound isolates and 22.2% (4/18) of urine isolates.

\textbf{Sequencing analysis of resistance genes encoding ESBL producers.} The sequencing analysis of the \textit{bla}_{\text{CTX-M}}, \textit{bla}_{\text{TEM}} and \textit{bla}_{\text{SHV}} types is revealed in Table IV. Out of the 20 \textit{bla}_{\text{CTX-M}} family members, the majority (75%; n=15) carried the \textit{bla}_{\text{CTX-M-15}} subtype. \textit{bla}_{\text{CTX-M-15}} was identified among \textit{K. pneumoniae} (n=7) and \textit{E. coli} (n=5) isolates. Out of the 18 \textit{bla}_{\text{TEM}} family members, TEM-1 was the most prevalent
variant (83.3%, n=15). This subtype was common among *A. baumannii* (n=7) and *K. pneumoniae* (n=5) isolates.

**Discussion**

The emergence of MDR patterns due to ESBL-producing *Enterobacteriaceae* and *A. baumannii* is becoming a global concern (13,31). The present study determined antibiotic susceptibility patterns and characterized ESBL genes among clinical isolates of *Enterobacteriaceae* and *A. baumannii*. Collectively, 63.1% of isolates exhibited MDR to different antibiotics. In addition, MDR patterns were identified in 63.5% of *K. pneumoniae* and 51.8% of *E. coli* isolates. In Riyadh, the capital of Saudi Arabia, MDR patterns were identified in 67% of *uropathogenic E. coli* isolates at a tertiary healthcare center (19). These values were higher than those reported in Libya, where the MDR phenotype was detected in 33.2% of *E. coli* and 42% of *K. pneumoniae* isolates from patients with urinary tract infections (32). The results were also consistent with the observed high prevalence of MDR patterns (100%) among clinical isolates of *Enterobacteriaceae* carrying ESBL resistance genes collected in Ethiopia (33).

In the present study, the overall resistance rates of MDR *Enterobacteriaceae* and *A. baumannii* were very high for most examined antibiotics, except for colistin. Previous studies have reported that co-resistance to several antibiotic classes of penicillins, cephalosporins, aminoglycosides, fluoroquinolones, trimethoprim/sulfamethoxazole and carbapenems was common among ESBL-producing Gram-negative bacteria (7,19,33,34). These findings indicated that the emergence of ESBL-producing microorganisms could cause susceptibility to various antibiotics.

A multiplex-PCR assay has been proposed to rapidly detect several resistance genes encoding ESBL-producing *Enterobacteriaceae* and *Acinetobacter baumannii*. ESBL, extended-spectrum β-lactamase.

**Figure 3. Prevalence and characterization of resistant genes encoding extended-spectrum β-lactamase-producing Enterobacteriaceae and Acinetobacter baumannii.** ESBL, extended-spectrum β-lactamase.
These results, coupled with the present findings, revealed that the prevalence of ESBL gene types can vary between locations and geographical regions. MDR ESBL-producing *K. pneumoniae* and *A. baumannii* have become common causes of healthcare-related infections (11,31). In the present study, the prevalence of *bla*<sub>TEM</sub> in *K. pneumoniae* was revealed to be 60.7%. Increasing rates of the *bla*<sub>TEM</sub> gene have been reported among clinical isolates of *K. pneumoniae* in Al-Qassim (70.9%) (8) and Riyadh (54.05%), in the Central region of Saudi Arabia (17). On the other hand, the frequency of *bla*<sub>TEM</sub> was high among *A. baumannii* isolates, which was consistent with a previous study from the Makkah city in the western region of the country (31). These high rates, which indicated the dissemination of such ESBL-producing isolates, is alarming for multiple hospitals. The high prevalence of the *bla*<sub>TEM</sub> gene detected in *K. pneumoniae* and *A. baumannii* isolates may increase the incidence rate of infection caused by these ESBL producers across different regions. This renders extensive surveillance studies in local and national hospitals in Saudi Arabia necessary to understand the transmission and epidemiology of resistance genes encoding ESBL-producing bacteria. However, using molecular methods in local hospitals to detect resistance genes may help develop effective

### Table III. Distribution of extended-spectrum β-lactamase resistance genes in Gram-negative isolates recovered from clinical samples of patients.

| Source of Gram-negative bacteria | Single gene | Combined genes |
|----------------------------------|-------------|----------------|
|                                  | *bla*<sub>TEM</sub> | *bla*<sub>CTX-M</sub> | *bla*<sub>OXA-1</sub> | *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> | *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> | Total |
| Sputum (n=30)                    | 22          | 3             | 1              | 3          | 1          | 30     |
| Urine (n=18)                     | 11          | 2             | 0              | 4          | 0          | 18     |
| Wound (n=13)                     | 7           | 3             | 0              | 3          | 0          | 13     |
| Blood (n=8)                      | 4           | 1             | 0              | 3          | 0          | 8      |
| Tracheal aspirate (n=2)          | 0           | 1             | 0              | 1          | 0          | 2      |
| Eye swab (n=1)                   | 1           | 0             | 0              | 0          | 0          | 1      |

### Table IV. Sequencing analysis results of *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes produced by *Enterobacteriaceae* members and *Acinetobacter baumannii*.

| ESBL gene | Total (n=40) | *K. pneumoniae* (n=13) | *E. coli* (n=12) | *P. mirabilis* (n=2) | *E. cloacae* (n=3) | *A. baumannii* (n=10) |
|-----------|--------------|------------------------|------------------|----------------------|--------------------|-----------------------|
| *bla*<sub>CTX-M</sub> subtype (n=20) |
| CTX-M-15  | 15           | 7                      | 5                | 1                    | 1                  | 1                     |
| CTX-M-71  | 1            | 0                      | 1                | 0                    | 0                  | 0                     |
| CTX-M-101 | 1            | 0                      | 0                | 0                    | 0                  | 1                     |
| CTX-M-127 | 1            | 0                      | 1                | 0                    | 0                  | 0                     |
| CTX-M-181 | 1            | 0                      | 0                | 0                    | 1                  | 0                     |
| CTX-M-182 | 1            | 0                      | 1                | 0                    | 0                  | 0                     |
| *bla*<sub>TEM</sub> subtype (n=18) |
| TEM-1     | 15           | 5                      | 2                | 0                    | 1                  | 7                     |
| TEM-115   | 1            | 0                      | 1                | 0                    | 0                  | 0                     |
| TEM-159   | 1            | 0                      | 0                | 1                    | 0                  | 0                     |
| TEM-169   | 1            | 0                      | 1                | 0                    | 0                  | 0                     |
| *bla*<sub>SHV</sub> subtype (n=2) |
| SHV-28    | 1            | 0                      | 0                | 0                    | 0                  | 1                     |
| SHV-226   | 1            | 1                      | 0                | 0                    | 0                  | 0                     |
new antimicrobial treatments against ESBL producers and improve the infection control system. In the present study, 

*E. coli* commonly produced the *bla*<sub>CTX-M</sub> gene (33.3%). Similarly, the predominance of *E. coli* carrying *bla*<sub>CTX-M</sub> genes has been reported in the western region of Saudi Arabia (39). Previous studies revealed that *bla*<sub>CTX-M</sub> is the most prevalent gene among uropathogenic *E. coli* isolates from patients with hospital and community-acquired infections (19,29). Furthermore, ESBL-producing *E. coli* collected from fecal colonization was revealed to produce the *bla*<sub>CTX-M</sub> gene as it has been reported in a previous study (40). Several factors and mechanisms contribute to the spread of bacterial clones carrying the *bla*<sub>CTX-M</sub> gene in Saudi Arabia, including plasmid dissemination and the clonal spread of bacterial strains, the frequent use of cephalosporins and the large population of migrant workers (13). According to Yasir et al (39), the high diversity in the *E. coli* clones may have arisen due to the fact that ~50% of the population of Saudi Arabia are expatriates from developing countries, including Pakistan, India, Bangladesh, the Philippines and African countries where self-medication in patients is evident.

The present study revealed that *bla*<sub>CTX-M-15</sub> was the most frequent subtype of the *bla*<sub>CTX-M</sub> type. This was consistent with data from Saudi Arabia (6,16,17) and several other parts of the world (1,2). These findings indicated that *bla*<sub>CTX-M-15</sub> is a public health concern, since it is the most widespread gene worldwide. The emergence of the *bla*<sub>CTX-M-15</sub> variant has been revealed to be attributed to the horizontal gene transfer of genetic elements and the clonal expansion of microorganisms (19,41). Furthermore, the widespread and unnecessary use of ceftriaxone and cefotaxime have contributed to the emergence and spread of *bla*<sub>CTX-M</sub> resistance genes (2).

Multiple ESBL resistance genes in a single bacterium render that strain more difficult to treat with several antibiotic drugs (16). In the present study, the co-existence of two different ESBL genes in the same strain was detected in 22.2% of isolates. However, the most common combination of ESBL resistance genes was between *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>, which was consistent with studies from Pakistan (42) and Algeria (12). However, the combined production of *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes was more frequently detected in *K. pneumoniae* (50%) and *E. coli* (28.6%) isolates. These figures were lower than those reported in Nepal, where two or more ESBL genes were present in 100% of *Klebsiella spp.* and 56.2% of *E. coli* clinical isolates from a teaching hospital (7). The value reported in the present study was considerably higher than the 3.4% reported in uropathogenic *E. coli* from the Eastern region of Saudi Arabia (29).

The present findings revealed that sputum was the most frequent source of various ESBL resistance genes in Gram-negative bacteria. This may be due to the several sputum samples collected from patients at intensive care unit (ICU) wards. A previous study indicated that the characterization of antibiotic susceptibility of bacterial pathogens from the sputum of patients in the ICU with ventilator-associated pneumonia can help control this type of infection (43). In addition, it is known that most patients admitted to the ICU are immunocompromised and/or undergoing invasive procedures, which would lead to prolonged antibiotic therapy (25). The extended stay, selective pressure and frequent use of antibiotic treatment of patients in the ICU contribute to the increase in ESBL producers (43).

The present study has several limitations that need to be addressed in future studies. Firstly, the study was laboratory-based; therefore, clinical data of the patients were not obtained to analyze the risk factors for ESBL infection and understand the epidemiological spread of ESBL genes. Secondly, the study was conducted in a single center in southern Saudi Arabia. The results can therefore not be representative of all parts of the southern region. Multicenter studies are required to confirm these findings. Thirdly, the AmpC β-lactamase class and other types of ESBL enzymes, (such as *bla*<sub>TEM</sub>, *bla*<sub>PER</sub>, *bla*<sub>GES</sub> and *bla*<sub>SHV</sub>) which confer significant antibiotic resistance among Gram-negative bacteria, were not examined.

In conclusion, the ESBL resistance genes were a significant cause of MDR patterns and conferred susceptibility to various antibiotic agents in *Enterobacteriaceae* and *A. baumannii*. The present study reported high levels of various resistance genes in ESBL-producing isolates, with *bla*<sub>TEM</sub> being the most prevalent type. In addition, the co-existence of two different ESBL genes has been frequently detected in a single bacterial pathogen (12,42). The *bla*<sub>CTX-M-15</sub> gene is the predominant variant among isolates carrying the *bla*<sub>CTX-M</sub> type. The emergence of various ESBL-resistant and coexisting genes in *Enterobacteriaceae* and *A. baumannii* is alarming and may significantly limit the efficacy of therapeutic options in hospital settings. However, extensive surveillance studies at both the local and national levels are urgently required to obtain an understanding of the transmission and epidemiology of resistance genes in ESBL-producing bacteria. Using molecular methods at local hospitals to detect resistance genes in ESBL-producing bacteria is recommended to improve the infection control system and help set effective antibiotic therapy plans.

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**Availability of data and materials**

All data generated or analyzed during the study are included in this published article. The datasets generated during the present study are available in the Genbank repository, (http://getentry.ddbj.nig.ac.jp/), accession numbers LC636038–LC636063.

**Authors’ contributions**

MEI, TBA, MA and BKE conceived the idea of the study and developed the protocol. MEI and MA designed and conducted the study. MEI and TBA analyzed and interpreted the data and wrote the initial draft. MEI, TBA, MA, BKE reviewed the literature. BKE revised the study for important intellectual
contents. MEI and BKE confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The Research and Ethics Committee at the College of Medicine, University of Bisha (Bisha, Saudi Arabia) reviewed and approved the present study protocol.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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