INTRODUCTION

Neonates are at a very high risk of developing life-threatening bacterial infections due to their under-developed immune systems, resulting in significant morbidity and mortality.\(^1\) The problem among these neonates in ICU settings is further complicated due to the use of broad-spectrum antibiotics, contact with healthcare workers (HCWs), and exposure to invasive and surgical procedures.\(^2\) In outbreak settings, NICU patients are particularly vulnerable to colonization and infection with pathogens such as multidrug-resistant...
Gram-negative bacteria. In the majority of cases, these healthcare-associated infections are caused by *Escherichia coli* and *Klebsiella pneumoniae* and require the administration of multiple antibiotics. *E. coli* is a common cause of community and healthcare-associated diseases, and over the past few decades, *E. coli* strains isolated from community-acquired infections have become increasingly resistant to antibiotics. Further, bloodstream infections caused by *K. pneumoniae* are also often reported in neonatal intensive care units (NICUs). Additionally, there has been rapid and global dissemination of extended-spectrum-β-lactamase (ESBL) producing *E. coli* and *K. pneumoniae* in hospital settings, which complicates and limits current antibiotic treatment options. This increase has been mainly due to the successful dissemination of CTX-M-15 gene-carrying mobile genetic elements. CTX-M-15, the most widely distributed of these enzymes, was first found in isolates of Enterobacteriaceae from India, but is now prevalent almost everywhere in the world. It appears to be associated with epidemic plasmids flanked with insertion sequences that facilitate easy spread and hyper production of β-lactamase, which may, in part, explain the gene’s rapid spread.

ESBL-producing *E. coli* and *K. pneumoniae* have previously been reported to cause outbreaks of infection in neonatal intensive care units (NICUs). However, to our knowledge, this is the first report of a CTX-M-15-positive *E. coli* and *K. pneumoniae* outbreak in the neonatal intensive care unit of a maternity hospital in Saudi Arabia. In this study, we retrospectively characterized 19-ESBL-positive *E. coli*, and 42-ESBL-positive *K. pneumoniae* isolates obtained from an outbreak of infection at a neonatal intensive care unit (NICU) in the Ha'il region of Saudi Arabia.

### Materials and Methods

#### Sample Collection

During April 2014, an outbreak of 3rd generation cephalosporin resistant bacterial infection was suspected within a neonatal intensive care unit in a maternity hospital at Ha’il, Saudi Arabia. As part of the investigations into the outbreak during a one-month period (April 2014), a total of 821 samples were screened, including 407 patient and 414 other samples (comprising healthcare workers, staff and swabs from the NICU environment). Extended-spectrum β-lactamase positive bacterial strains of *E. coli* and *K. pneumoniae* cases were defined as those patients admitted to the NICU from whom at least one sample recovered during the NICU stay contained an ESBL producer. Only one non-repetitive

### Table 1 Specific PCR Primer Pairs Used in This Study.

| Resistance Type | Primer Name | Primer Sequence (5'- 3') |
|-----------------|-------------|--------------------------|
| **blaTEM**      | TEMFU       | TCGTGTCGCCCTATTCCTTTTT   |
|                 | TEMRU       | GCGCGAGTTAGCTCCTCGCGTCCTC |
|                 | TEMFLF      | GAAGACGAAAGGGCTCGTG      |
|                 | TEMFLR      | GTCTGAGATGTCATACCAATGC   |
| **blaSHV**      | SHVFU       | GGGATGCGGCTGACGACAGC     |
|                 | SHVRU       | TGCCGAAAAAGGCAGTCAATCTT |
|                 | SHVFLF      | CGCGGGTTATCTTATTTTTCG   |
|                 | SHVFRL      | TCTTCCGATGCGGCGCAGTC   |
| **blaCTX-M**    | CTX-MUF     | CGCTTTGCGATGTGCAG        |
|                 | CTX-MUR     | ACCGGCAGTATGGTTG         |
|                 | CTX-M gP1F  | AAAATCAGCTGCGCCAGTTCC    |
|                 | CTX-M gP1R  | AGCTTTATCATGCGACATT      |
|                 | CTX-M gP2F  | CGACGCATCCCTGCTATTT      |
|                 | CTX-M gP2R  | CAGCGCGATGCAATTCGAGG     |
|                 | CTX-M gP9R  | AAAAGAGAGTGCAACCGATG     |
|                 | CTX-M gP9R  | ATGGAAAGCGTCTACACC       |
|                 | CTX-M gP82R | TCGCGTTAAGGCGATGAGC      |
|                 | CTX-M gP2SF | AACCAGCGATGCGGTAGC       |
|                 | CTXMSSeqF   | GCACTGACATCCGGG          |
|                 | CTXMSqetR   | GTCTGCTTCCCAAATGAGG      |
| **Note:** Adapted from Hassan H, Abdalhamid B. Molecular characterization of extended-spectrum beta-lactamase producing Enterobacteriaceae in a Saudi Arabian tertiary hospital. J Infect Dev Ctries. 2014;8(3):282–288. doi:10.3855/jidc.3809.14
ESBL-positive bacterial isolate was processed from each ESBL-positive patient.

**Bacterial Identification and Antibiotic Susceptibility Testing**

The cultured bacterial isolates were identified by a routine culture-based identification system, as well as MALDI-TOF mass spectrometry (Bruker Daltonics-Germany). ESBL’s were initially screened for reduced susceptibility to cefpodoxime, cefotaxime, ceftiraxone, ceftazidime, or aztreonam, and then by performing phenotypic confirmatory test by demonstrating a synergistic effect between an indicator cefalosporin and a β-lactamase inhibitor. The ceftazidime (30 µg) discs alone and in combination with clavulanic acid (ceftazidime + clavulanic acid, 30/10 µg discs) were applied onto a plate of Mueller Hinton Agar (MHA), which was inoculated with the test strain. An increase of ≥ 5 mm in the zone of inhibition of the combination discs in comparison to the ceftazidime disc alone was considered to be a marker for ESBL production. Additional antibiotic susceptibility profiling of the positive isolates was then performed using the MicroScan WalkAway-40 plus (Indianapolis, United States) system.

**DNA Extraction**

Bacterial DNA was as isolated using the automated QIAcube device and the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

**Identification of β-Lactamase Genes**

Genes encoding for β-lactamase enzymes (blaCTX-M, blashv, and blatem) were detected using PCR and sequencing according to a previously published method. The specific primer pairs that are used in the current study are shown in Table 1.

**Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST)**

PFGE was performed using XbaI-digested fragments of bacterial chromosomal DNA, with fragment separation achieved in 0.8% agarose. Electrophoresis conditions comprised a constant voltage of 6 V/cm at 148°C and pulse times of 3.5–25 s increased linearly over 12 h (block 1), followed by 1–5 s increased over an 8-hour period. Gel patterns were analyzed using BioNumerics software (Applied Maths) with the band tolerance set at 1.0%.

Multilocus sequence typing was performed using seven housekeeping genes in *E. coli* and *K. pneumoniae*.16,17

**Results**

**Prevalence and Susceptibility Patterns**

A total of 24 *E. coli* isolates were cultured from neonates, with the majority of isolates (19/24) being ESBL-positive (Figure 1) and resistant to 3rd generation cephalosporins. However, all of the isolates were susceptible to aminoglycosides (amikacin, gentamicin and tobramycin), except for 2 isolates that were intermediate resistant to tobramycin. All isolates were also susceptible to cefoxitin, ciprofloxacin, levofloxacin and imipenem. Additionally, 48 *K. pneumoniae* isolates were also cultured from neonates, with the majority of isolates 87.5% (42/48) being ESBL positive (Figure 2) and resistant to 3rd generation cephalosporins. Overall 90% (37/42) of these ESBL-positive *K. pneumoniae* isolates were resistant to gentamicin and tobramycin but all 42 (100%) isolates were susceptible to amikacin. However, all of the *K. pneumoniae* isolates were susceptible to ciprofloxacin, levofloxacin and imipenem. None of the umbilical isolates from neonates (*E. coli* or *K. pneumoniae*) were cultured from neonates with omphalitis (inflammation of the umbilical cord stump).

**Characterization of β-Lactamase Genes**

Characterization of β-lactamase genes among *E. coli* isolates revealed that 100% (19/19) of ESBL-positive *E. coli* isolates were found to harbor the CTX-M-15 gene. Further, 15% (3/19) of *E. coli* isolates possessed both CTX-M-15 and SHV-12 genes, with 68.4% (13/19) being TEM-1 positive (Figure 1). Among *K. pneumoniae*, 92.85% (39/42) of isolates contained the CTX-M-15 gene. A total of 95% (38/40) of *K. pneumoniae* possessed both the CTX-M-15 and SHV-12 genes. Further, 88% (38/42) were positive for TEM-1 (Figure 2).

**PFGE and MLST Genotyping**

PFGE result showed that the majority of *E. coli* isolates grouped into 2 genetic clusters at 80% similarity (18/19 isolates) and 12 genotypes at 95% similarity. The majority (31/42) of *K. pneumoniae* isolates belonged to a single genotypic lineage at the 85% similarity level and 16 genotypes at the 95% similarity level.

MLST results showed that all 19 *E. coli* isolates belonged to ST131 and all *K. pneumoniae* isolates belonged to ST 14.
Discussion

Our results indicate some interesting features of retrospective E. coli and K. pneumoniae isolates associated with NICU infections in Saudi Arabia. For example, a 2-year study of a neonatal intensive care unit in Riyadh, Saudi Arabia in 2000 already indicated that E. coli was the most common infecting organism associated with early onset sepsis. Additionally, E. coli and K. pneumoniae have been reported as the most commonly isolated bacteria associated with neonatal septicemia in Saudi Arabia (Riyadh) at least as far back as the 1980’s. Additionally, E. coli and K. pneumoniae have been reported as the most commonly isolated bacteria associated with neonatal septicemia in Saudi Arabia (Riyadh) at least as far back as the 1980’s.  

Within Saudi Arabia, the dominance of ST131 E. coli isolates in population-based studies has previously been reported, extending back to at least 2011. Further, this clonal lineage of E. coli continues to be problematic in Saudi Arabia, although infections associated with the ST131 clone are not only isolated to Saudi Arabia. Additionally, associations between E. coli ST131 clonal type and carriage of the CTX-M-15 gene on IncFIA F2:A1:B-] (Group 2) plasmids of Clade 2 isolates has been reported. CTX-M-15 positive E. coli and K. pneumoniae have been reported to cause outbreaks in neonatal intensive care units (NICUs) worldwide. Additionally, K. pneumoniae ST14 isolates expressing CTX-M-15 have been reported as being associated with neonatal sepsis in Tanzania between 2009 and 2010, as well as in Spain in 2008.

In Saudi Arabia, several studies have reported the dominance of CTX-M-15 genes among Enterobacteriaceae, including in K. pneumoniae isolates associated with an NICU in 2007. In fact, among CTX-M genes, CTX-M-15 is the most prevalent reported worldwide. In CTX-M-15-positive Enterobacteriaceae isolates from Riyadh in 2009 were associated with a high prevalence of quinolone resistance, although our isolates were all quinolone susceptible. This may reflect differences in antibiotic prescribing practices between different cities within Saudi Arabia. The threat posed by multi-drug resistant K. pneumoniae to the Arabian gulf countries was recently highlighted by Bindayna, Joji, Ezzat and Jahrami in a meta-analysis of 28 published articles reported a high prevalence of blaOXA-
and bla_{CTX-M}, followed by bla_{SHV}, bla_{TEM}, bla_{NDM-1} and bla_{VIM} genes in *K. pneumoniae*. Unfortunately, the consequences of such multi-drug resistance to citizens of Saudi Arabia are not a recent phenomenon, as indicated by the current publication. In 2019, a 33-month surveillance study in three pediatric and neonatal intensive care units in Riyadh concluded that aminoglycosides represented 45.4% of monitored antimicrobials used in neonatal ICU followed by cephalosporins (30.4%). Prescribing practices may vary between hospitals, but this information could indicate why combined aminoglycoside and cephalosporin (*K. pneumoniae*) and cephalosporin (*E. coli*) antimicrobial resistance is extensively observed in our results. Videoconventional mechanical ventilation and total parenteral nutrition were previously identified as significant risk factors for nosocomial infection within a neonatal care unit in South-Western Saudi Arabia. Other risk factors associated with neonatal sepsis from Riyadh (2011–2015) were prematurity, as well as multiparity and delivery by caesarean section. Similar to the current publication this same report indicated that all Gram-negative organisms isolated were sensitive to amikacin. It has previously been reported that the carriage of the bla_{SHV}-related genes may be universal in *K. pneumoniae* isolates, while three *K. pneumoniae* isolates from our collection were repeatedly SHV negative using PCR. Various technical issues (DNA extraction, PCR thermocycling, etc.) or biological factors (geographical location, nucleotide changes in primer binding sequences, etc.) could have been responsible for this discrepancy. However, further investigations are beyond the scope of this publication and the negative results do not influence the authors’ conclusion.
Conclusions
This is the first report of CTX-M-15-positive, ESBL E. coli and K. pneumoniae isolates recovered from an outbreak in an NICU in Ha’il, Saudi Arabia. It is alarming to note the high rate of outbreak isolates with simultaneous production of CTX-M-15 and SHV-12 conferring high-level resistance to oxyimino-cephalosporins, even in isolates cultured in 2014. Further studies are required in order to indicate the extent and spread of CTX-M-15-positive Enterobacteriaceae in more recent years within Saudi Arabia.

Ethical Approval
There was no approval required for this research by an institutional review board or ethics committee.

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Disclosure
The authors report no conflicts of interest in this work.

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