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Multi-drug carbapenem-resistant *Klebsiella pneumoniae* infection carrying the OXA-48 gene and showing variations in outer membrane protein 36 causing an outbreak in a tertiary care hospital in Riyadh, Saudi Arabia

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SUMMARY

Objectives: To investigate the genes of antibiotic resistance among isolates from the first reported carbapenem-resistant *Klebsiella pneumoniae* (CRKP) outbreak in a tertiary care hospital, Riyadh, Saudi Arabia.
Methods: Antimicrobial susceptibility testing was performed on bacterial isolates using the Microscan Walkaway system (Siemens, Germany) and was confirmed by Etest (AB Biodisk, Sweden). *bla*$_{-CTX-M}$, -SHV, -TEM, -OXA-48, OXA-A,B,C,D, -KPC, -NDM, -VIM, -IMP, integron 1, and outer membrane proteins(Omp)-35 and Omp-36 were investigated by PCR amplification and direct sequencing of PCR products. Isolates were sequence-typed by multilocus sequence typing (MLST).
Results: All isolates were resistant to cefotaxime, ceftazidime, cefepime, ciprofloxacin, and piperacillin–tazobactam, and 91% (21 out of 23) were resistant to amikacin and gentamicin. All isolates except two from a single patient were resistant to one of the carbapenems. CTX-M and SHV genes were detected in all isolates, CTX-M-15 and SHV-1 types being predominant among these extended-spectrum beta-lactamases (ESBLs). TEM-1 was found in all except one isolate (isolate 3). Significantly, the OXA-48 gene was also found in all isolates. OXA-D-gene was found in three out of 23 isolates. KPC, NDM, OXA-A, -B, -C, VIM, and IMP genes were absent in all isolates. Disruption of the Omp-36 gene due to insertion of transposon IS903 and/or IS4 was detected in four out of 23 isolates, and some unique variations were also observed in this gene, including an insertion of two amino acids in the L3 region of Omp-36 in one isolate (isolate 3) and a mutation resulting in a premature stop codon in another isolate (isolate 25). MLST revealed ST29 to be the predominant sequence type (17 out of 23 isolates, 74%). Three were ST709 and one each was ST37 and ST111; one isolate had an unknown ST.
Conclusions: This is probably the first reported outbreak of multidrug/carbapenem-resistant Klebsiella infection involving the OXA-48 gene from Saudi Arabia. Although the presence of ESBLs such as OXA, CTX-M, TEM, and SHV are predictable reasons for resistance, variations in the Omp-36 gene might also have precipitated this phenomenon. Disruption of the Omp-36 sequence by large insertional elements, the insertion of two amino acids in a very crucial part of this protein, and the presence of a premature stop codon in one isolate might have rendered this protein incomplete and non-functional. The study also demonstrated that more than one type of clone was responsible for this reported apparent outbreak and that ST29, a clone not reported from this region before, was the major clone responsible.

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1. Introduction

Klebsiella pneumoniae is a pathogen of the *Enterobacteriaceae* family that causes healthcare-associated infections and has recently emerged as one of the most antibiotic-resistant organisms responsible for outbreaks in the healthcare setting.[1,2] Although *Enterobacteriaceae* members are known to produce the conventional extended-spectrum beta-lactamases (ESBLs) conferring resistance to different types of antibiotics, carbapenem resistance was previously uncommon in *K. pneumoniae* isolates.[3] However, a significant number of emerging reports suggest that the susceptibility to carbapenem of these bacteria is no longer guaranteed.[4] Resistance to carbapenem in these organisms is on the increase and this poses a significant threat in the management of multidrug-resistant isolates.[5]

Carbapenem resistance can arise through the acquisition of resistance genes encoding metallo-beta-lactamases, non-metallo-carbapenemases (KPC, GES, or OXA-type), AmpC, or ESBLs and an alteration in the expression of the outer membrane protein (OMP).[6–8] Resolution of the three-dimensional structure of the *Escherichia coli* OmpF and *K. pneumoniae* OmpC-like porin (OmpK36) has led to the identification of the functional domains of the channels.[9] Recent studies have identified amino acids important in porin structure and function in bacteria.[10] The replacement of these amino acids by mutation may greatly decrease diffusion through the porin in some clinical strains.[11] The mutations detected in L5, L6, and in the strategic L3 have been implicated in marked resistance to cephalosporins and impaired cephalosporin uptake associated with a decrease in ion conductance (De E et al., 2001). The involvement of porin loss in combination with the conventional ESBL genes and KPC in a carbapenem-resistant *K. pneumoniae* outbreak has recently been reported from a Chinese hospital.[12] Here we report our findings on the occurrence of newly described OXA-48, the distribution of different ESBLs, and some unique variations in Omp-36 in isolates from an outbreak of carbapenem-resistant *K. pneumoniae* infection at King Abdulaziz Medical City, a tertiary care hospital in Riyadh, Saudi Arabia.

2. Methods

Isolates saved from the 2010 outbreak were identified by the hospital microbiology laboratory, as detailed in our previous report.[13] During this period, a total number of 23 isolates were collected from 22 patients in different wards. Routine microbiological investigations including minimum inhibitory concentration (MIC) determinations were carried out. These isolates were stored in trypticase soy broth (TSB) with 20% glycerol at −70 °C and revived on sheep blood agar plates; they were then transferred to TSB and incubated overnight at room temperature at the time of use for further studies.

2.1. Antibiotic susceptibility tests

Isolates were identified using the standard laboratory methods of the Clinical and Laboratory Standards Institute (CLSI) guidelines.[14] Identification of *K. pneumoniae* to the species level was done using the Microscan Walkaway system (Siemens, Germany) and then confirmed using the API20E Bacterial Identification System (bioMérieux, USA). Antimicrobial susceptibility testing was determined using the Microscan Walkaway system and confirmed using the Etest (AB Biodisk, Sweden). MIC breakpoints for carbapenems (meropenem and/or imipenem) were defined according to the 2010 CLSI guidelines.[15] *K. pneumoniae* isolates found to have elevated MICs for carbapenem were tested for the presence of carbapenemases using the modified Hodge test[16] as part of our investigation into the outbreak; details are given in our previous publication.[13]

2.2. Multilocus sequence typing (MLST)

MLST was done on these isolates using the protocol of Diancourt et al.[17] Briefly, the seven conserved housekeeping genes, namely *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*, were PCR amplified and sequenced. The sequences were submitted to the KP-MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/primers_Kpneumoniae.html) for analysis and sequence type (ST) designation.

2.3. ESBLs and other related genes

The primers used for PCR amplification for ESBLs and other related genes and their subsequent sequencing are listed in Table 1. A uniplex PCR was run for all genes under study. All results are based on a positive PCR amplification and subsequent sequencing of each gene, at least in duplicate, for all of the isolates. A PCR was considered negative after failure to amplify the target of expected size at least three times. Internal primers were designed and utilized to fully characterize the insertion elements detected in some of the isolates and for full-length sequencing of Omp. The amplicons of positive reactions were purified using MinElute PCR/Gel purification columns (Qiagen). Sequencing was performed in both directions on an ABI-3100 DNA Analyzer. The sequences were analyzed using the SeqMan (Lasergene 6) software tool. Variation in any nucleotide was taken into consideration only when it was observed in sequences of both directions. Sequences thus obtained were submitted to BLAST for determination of identities in the GenBank sequence database of the National Center

Table 1
List of primers used in this study for various gene targets

Gene target	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Reference
KPC	GATACCACGTTCCGTCTGG	GCAGGTTCCGGTTTTGTCTC	Hindiyeh et al.[34]
VIM	TTTGGTCGCATATCGCAACG	CCATTCAGCCAGATCGGGCAT	Hujer et al.[35]
IMP	GTTTATGTTCATACATCG	GGTTTAACAAAACAACCAC	Hujer et al.[35]
OXA-48	TTGGTGGCATCGATTATCGG	GAGCACTTCTTTTGTGATGGC	Poirel et al.[36]
OMP-35	CAGACACCAAACTCTCATCAAGGG	AGAATTGGTAAACGATACCCACG	Kaczmarek et al.[25]
OMP-36	CAGCACAATGAATATAGCCGAC	GCTGTTGTCGTCCAGCAGGTTG	Kaczmarek et al.[25]
OMP-36 N	CTGCGGCTGACCTGTCGCTGAAC	CGGTCAGCTGGTCGTTGATCTGG	This study
SHV	ATGCGTTATATTCGCCTGT	TGCTTTGTTATTCGGGCCAA	Hujer et al.[35]
TEM	AAACGCTGGTGAAAGTA	AGCGATCTGTCTAT	Paterson et al.[37]
CTX-M	TTTGCGATGTGCAGTACCAGTAA	CGATATCGTTGGTGGTGCCATA	Edelstein et al.[38]
PER	ATGAATGTCATTATAAAAG	TTGGGCTTAGGGCAG	Hujer et al.[35]
INTEGRON-1	TCATGGCTTGTTATGACTGT	GTAGGGCTTATTATGCACGC	Hujer et al.[35]

Table 2
Minimum inhibitory concentrations (μg/ml) of antimicrobial agents tested for the outbreak isolates of *Klebsiella pneumoniae*

Isolate No.	Cefotaxime	Ceftazidime	Cefepime	Gentamicin	Amikacin	Ciprofloxacin	TMP–SMX	Meropenem	Ertapenem	Imipenem	TZP
1	>16	>32	64	64	96	6	32	4	16	6	256
2	>16	>32	256	1	256	>32	>32	>32	>32	>32	256
3	64	256	16	0.25	48	256	0.064	5	32	12	256
4	256	256	256	32	64	256	32	32	32	32	256
5	>16	>32	256	128	256	>32	32	>32	>32	>32	256
6	256	256	256	8	64	32	2	32	32	32	256
7	>16	>32	256	32	64	>32	>32	>32	>32	>32	256
8	256	256	256	6	32	32	0.5	6	32	8	256
9	256	256	256	32	4	8	32	8	32	24	256
12	256	64	192	256	128	32	32	0.64	0.64	0.125	32
13	256	64	192	256	128	32	32	0.64	0.64	0.125	32
14	256	>32	16	24	32	32	>32	3	4	0.75	256
16	256	192	256	16	32	12	3	16	32	32	256
17	256	128	48	8	16	16	0.19	4	16	4	256
18	>16	>32	256	64	96	4	>32	4	>32	1.5	256
19	256	96	64	16	32	32	32	3	4	4	256
20	256	128	32	24	64	6	32	1.5	6	1	256
21	256	16	24	24	32	4	32	4	8	0.5	256
22	256	256	256	4	2	32	32	32	32	32	256
23	N/A	N/A	256	256	128	32	N/A	8	N/A	8	N/A
24	256	192	256	64	256	32	32	32	32	32	256
25	256	128	256	48	128	16	32	32	32	32	256
26	256	192	256	32	256	32	32	4	4	4	256

TMP–SMX, trimethoprim–sulfamethoxazole; TZP, piperacillin–tazobactam; N/A, Not Available.

for Biotechnology Information (NCBI, USA). Deduced protein sequences for OMPs were aligned against the reference sequences using BioEdit sequence alignment software. Phylogenic and molecular evolutionary analysis was conducted implementing the Kimura 2-parameter model using MEGA version 4.[18]

3. Results

3.1. MICs

The MIC results for the isolates are given in Table 2. As per the criterion for a multidrug-resistant organism (MDRO), all isolates were resistant to three or more types of antibiotics. All isolates were resistant to cefotaxime, ceftazidime, cefepime, ciprofloxacin, and piperacillin–tazobactam, whereas 91% (21 out of 23) were resistant to amikacin and gentamicin. Two isolates from a single patient were sensitive to carbapenems but resistant to the rest of the antibiotics.

3.2. MLST

The MLST results showed four sequence types (STs) to be involved in this outbreak. Out of 23 isolates, 17 were ST29. Isolates 6, 8, and 22 belonged to ST709. Isolate 3 was ST111, whereas isolate 2 was ST37. The ST for isolate 19 did not match any of the types in the database, however ST111 was the nearest match.

Table 3
ESBLs and other genes in isolates from the reported outbreak

Isolate No.	MLST	Index date	OXA-48	OXA- D	TEM-1	SHV-1	SHV-11	SHV-12	CTX-M-14	CTX-M-15	Class 1 integron
1	29	Mar 24, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
2	37	Mar 5, 2010	Pos	Neg	Pos	Neg	Pos	Neg	Neg	Pos	*aadB, dfrA7*
3	111	Aug 21, 2010	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos	*aadB, dfrA17*
4	29	Mar 31, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
5	29	Mar 28, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
6	709	Jul 7, 2010	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Pos	-
7	29	April 6, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
8	709	Aug 22, 2010	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Neg	-
9	29	May 8, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
12	29	Dec 1, 2009	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB, dfrA7*
13	29	Dec 1, 2009	Pos	Neg	Pos	Pos	Neg	Neg	Pos	Neg	*aadB*
14	29	Mar 27, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
16	29	Jul 22, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	-
17	29	Jul 15, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	-
18	29	Feb 14, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	-
19	UK[a]	Mar 6, 2010	Pos	Neg	Pos	Neg	Pos	Neg	Neg	Pos	*aadB*
20	29	Feb 26, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	-
21	29	Mar 3, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*dfrA7*
22	709	Mar 13, 2010	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Neg	*aadB*
23	29	Mar 9, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
24	29	Jun 16, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
25	29	Apr 12, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*
26	29	Jul 18, 2010	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Pos	*aadB*

MLST, multilocus sequence typing; UK, unknown; Pos, Positive; Neg, Negative.
[a] Nearest match is ST111.

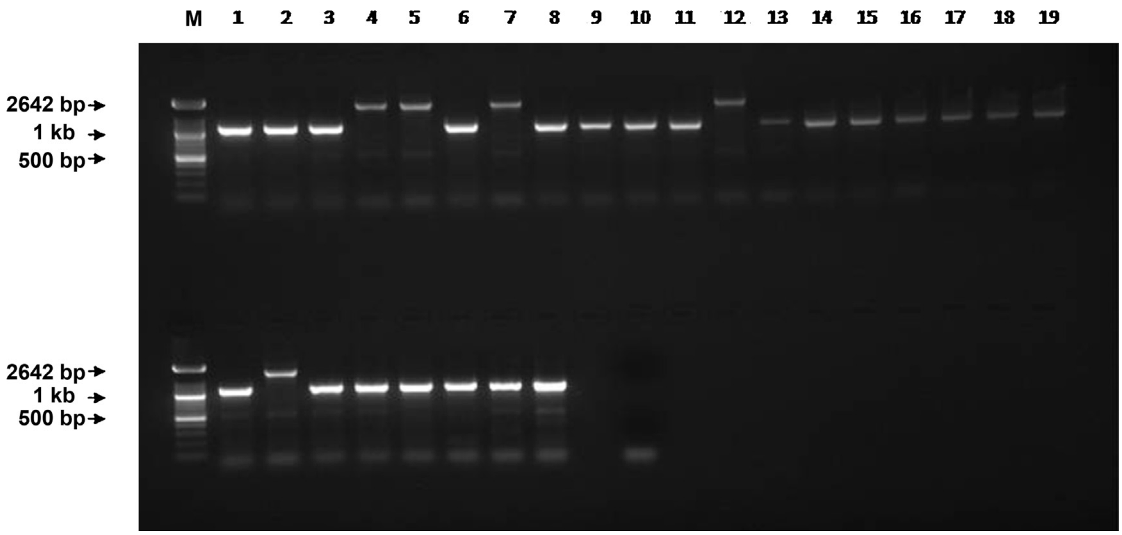

Figure 1. PCR amplification of the Omp-36 gene in Klebsiella isolates. Upper row: M, 100-bp marker; lanes 1–9 are isolates 1–9; lanes 10–12 are isolates 12–14; lanes 13–19 are isolates 16–22. Lower row: M, 100-bp marker; lanes 1–4 are isolates 23–26; lanes 5–8 are positive control strains of Klebsiella; lane 9 is empty; lane 10 is the negative control. Lanes 4, 5, 7, and 12 of the upper gel and lane 2 of the lower gel show an insert in this gene.

3.3. Detection of ESBLs and other resistance genes

The results of the antibiotic resistance genes are given in Table 3. OXA-48 was present in all isolates. OXA-D-type gene was found in three isolates. CTX-M and SHV genes were also found in all isolates, CTX-M-15 and SHV-1 being predominant among these ESBLs (87% and 74%, respectively). TEM-1 was found in all except one isolate (isolate 3). Class 1 integron was

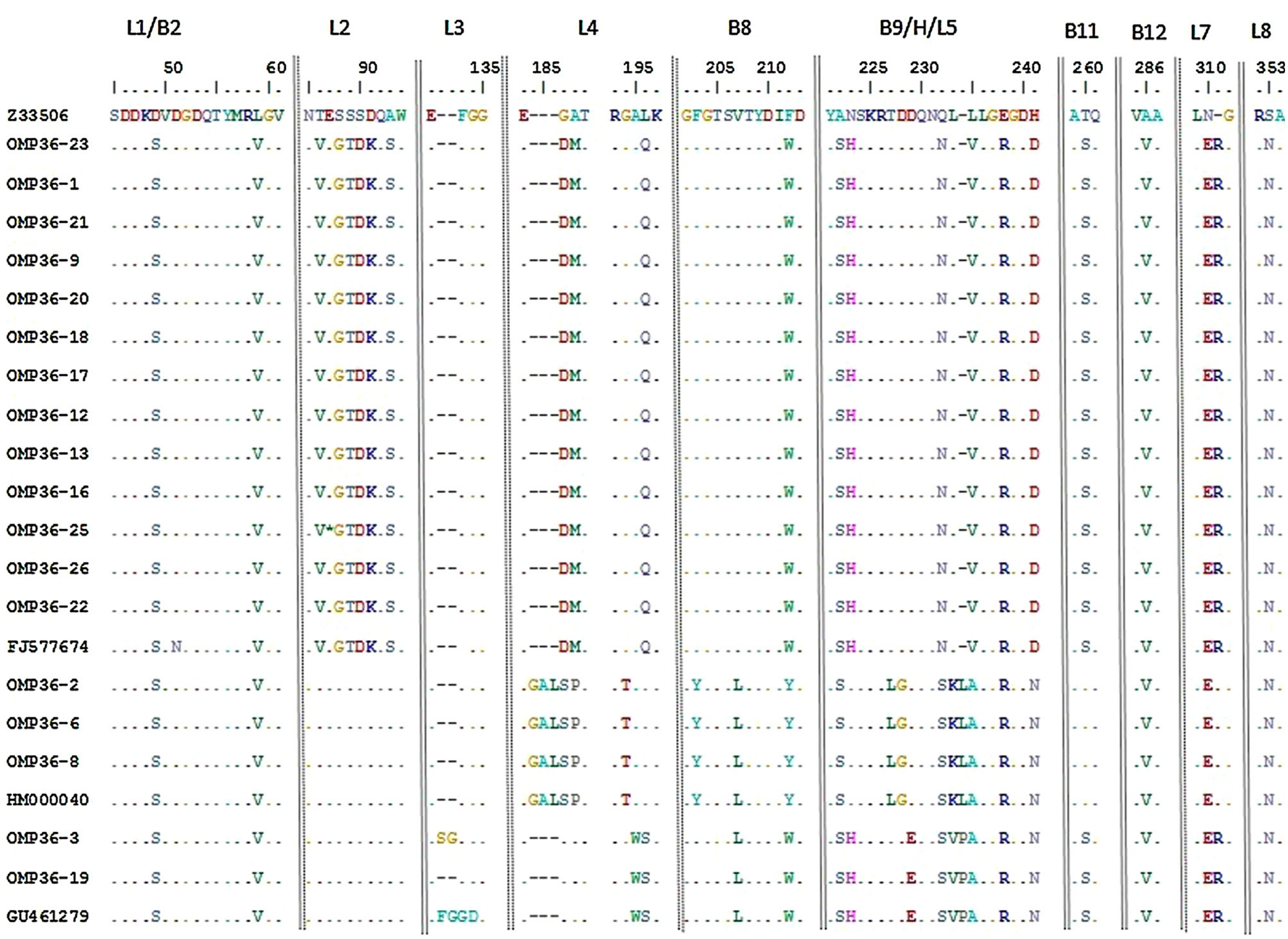

Figure 2. Omp-36 protein sequence alignment for Klebsiella isolates with some known sequences. Only those parts that showed a substitution, insertion, or a deletion are presented. A sequence from GenBank (Z33506) was used as the reference sequence.

positive in 74% of isolates and *aadB* was predominant. None of the isolates was positive for OXA-A, -B, -C, KPC, NDM, VIM, or IMP.

3.4. Outer membrane protein sequence analysis

A normal Omp-35 sequence was observed in all isolates. However, large numbers of variations were found in the Omp-36 gene sequence. Four isolates (4, 5, 7, and 14) had an amplicon larger than the expected size (approximately 1.1 kb) for Omp-36 (Figure 1). Upon sequencing, these isolates were found to have an insertion of transposon IS903 at nucleotide 127 of their Omp-36 gene. Isolates 4 and 5 had an insert of 1069 bp, whereas the size of the insert in isolates 7 and 14 was about 1220 bp, with a difference of 163 bp. Of these 163 nucleotides, 127 were a repeat sequence of the beginning of Omp-36, whereas the remaining 36 nucleotides were from IS4 insertion element. The 19 remaining isolates with no insert also showed a large number of variations in their Omp-36 gene. These variations included additions, deletions, and substitutions when compared to wild-type *K. pneumoniae* strain ATC 13883 (accession number **Z33506**). These variations are shown in Figure 2. A phylogenetic relationship based on Omp-36 sequence similarity of these isolates with the known sequences is depicted in Figure 3.

4. Discussion

Many of the carbapenem-resistant *Enterobacteriaceae* outbreaks identified have been related to the production of carbapenemases, specifically KPC-1 and -3, as well as the metallo-beta-lactamases VIM and IMP.[19] However, resistance to carbapenem is not restricted to the above and may involve other mechanisms, including structural changes in the outer membrane and hyper production of AmpC, in addition to ESBL or carbapenemase production.[1,19,20] The observation of the newly described OXA-48 in all *K. pneumoniae* isolates in our study is significant. This is probably the first report of a carbapenem-resistant *K. pneumoniae* outbreak in a hospital setting in Saudi Arabia involving OXA-48, acknowledging the recent findings of Mathers et al. in a patient who had travelled from Riyadh, Saudi Arabia and was reported to be infected by *K. pneumoniae* with OXA-48.[21] This may suggest a more serious endemicity of this gene among *K. pneumoniae* isolates from the hospital setting in the Kingdom or even the Gulf countries, and is worthy of further epidemiological studies.

The high incidence (87%) of isolates with co-expression of OXA-48 and CTX-M-15 found in this study has also been reported widely from other parts of the world.[5,22,23] The presence of CTX-M-15 and SHV-12 as predominant ESBLs in *K. pneumoniae* isolates in this study is consistent with the findings of another recently reported study from this region.[24] There also appears to be a correlation between the sequence type and an ESBL genotype, particularly the *bla*-SHV and *bla*-CTX-M subtypes. Among non-ESBL SHVs, SHV-1 was found in isolates with ST29 and SHV-11 in those with ST111/37, whereas ESBL-positive SHV (SHV-12) was seen in isolates with ST709. However, this could be due simply to the clustering of isolates in an apparent outbreak. The CTX-M-15 gene was found in the majority of isolates (20 out of 23 isolates; 86.9%) regardless of the sequence type, whereas two out of three isolates exhibiting CTX-M-14b were ST709.

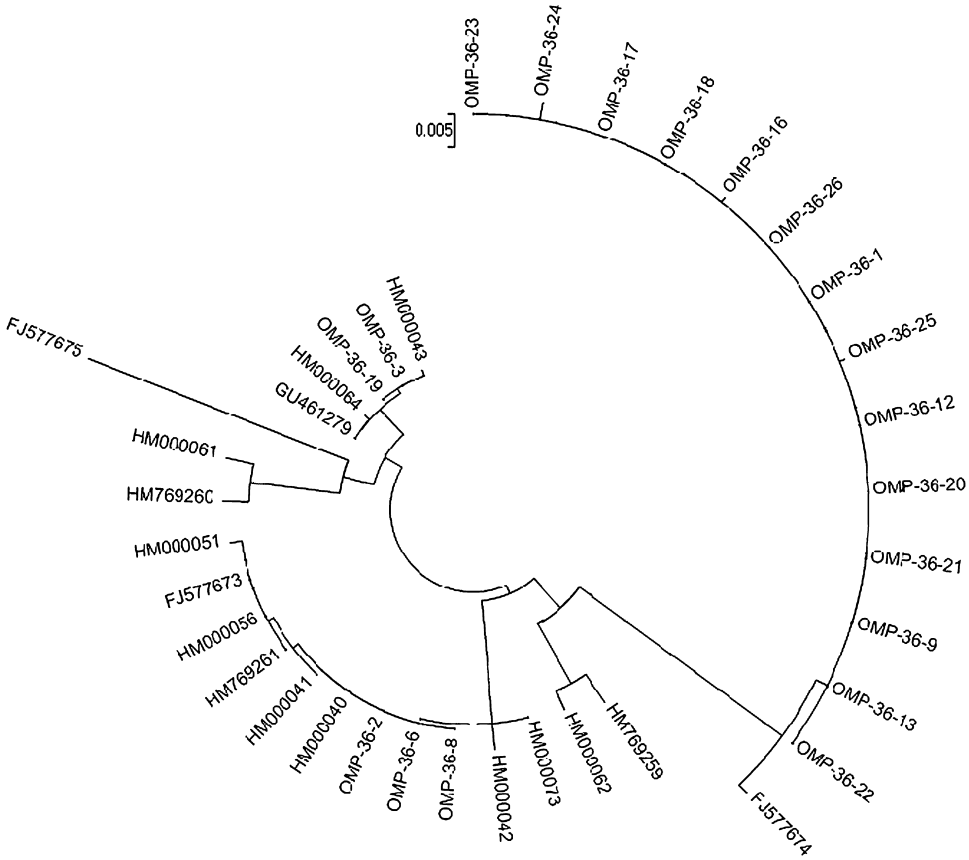

Figure 3. Phylogeny of isolates from this study based on Omp-36 sequence similarities. These isolates are clustered in three clusters, the largest comprising 14 isolates and matching with **FJ577674**.

The expression of Omp-36 has been shown to be a major factor in conferring resistance against carbapenems in *K. pneumoniae*.[7] The disruption of Omp-36 by virtue of insertional elements (IS903 and IS4), as seen in isolates 4, 5, 7, and 14 from our outbreak, has been reported previously in carbapenem-resistant *K. pneumoniae* isolates from Westchester Medical Center, NY[25] and Taiwan.[26] The G→T mutation observed in isolate 25 at nucleotide position 259 generates a TAA stop codon in the sequence, thus resulting in an incomplete or non-functional OMP protein. The unique variations observed in the cluster comprising 13 isolates (Figure 2) had not been described previously in a *K. pneumoniae* strain with known biological properties. The hot spot for variations in these isolates is in the region of L2 and in the L4–L5 regions (Figure 2). It has been reported that L2 has an affinity to bind with imipenem and a deletion in this region has resulted in much lower affinity to imipenem.[27] A crucial variation in the L3 region in isolate 3 was observed in the form of insertion of serine and glycine residues in the highly conserved motif PEFGGD (Figure 2); this has not been reported previously, although the addition of two different amino acids (phenylalanine and glycine) was observed in a Klebsiella strain NVT2001, serotype 2 isolated from a patient with liver abscess in Taiwan.[28] A mutation of G→D at L3 and variations in other regions including L4 and L5 of Omp-36 have widely been shown to cause major functional changes in the bacterial porin function, including a decrease in carbapenem and cephalosporin diffusion through it.[11,29–31] Functional studies are needed to confirm the effects of these variations; however, it has been shown that there could be an interplay between different mechanisms to achieve carbapenem resistance.[32]

The sequence typing by MLST on these isolates showed that the largest group (17 out of 23) involved in this outbreak belonged to ST29, a genotype not reported from this region before. The other STs involved were ST709 with three isolates and ST111 with two isolates. An allelic difference was observed at locus *InfB* due to a single nucleotide variation in one of these two ST111 isolates. ST111 was reported in a human patient from Spain in 1993 and was subsequently repeatedly isolated from bovine origin samples in the USA.[33] The much more common sequence type ST37 was found in only one patient.

In conclusion, this report presents an outbreak of multidrug/carbapenem-resistant *K. pneumoniae* carrying OXA-48 in combination of CTX-M-15. To our knowledge no outbreak involving OXA-48 has been reported from Saudi Arabia before. It also appears that the outer membrane porin Omp-36 plays a contributing role in antibiotic resistance. ST29, the major clone responsible for the present outbreak, is new to this region and requires further investigation.

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Conflict of interest: All authors declare no conflicts of interest relevant to this article.

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