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A point-prevalence survey of carbapenem-resistant Enterobacteriaceae in two different cities in Kuwait and Nigeria

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Abstract:

Background: The family Enterobacteriaceae belongs to the order Enterobacterales, a large diverse group of Gram-negative, facultatively anaerobic bacteria that sometimes cause multidrug-resistant infections which treatment options are often challenging. They are the leading cause of nosocomial bloodstream infection (BSI) and urinary tract infections (UTI). The objective of the study was to carry out a point-prevalence survey of antimicrobial resistance and carbapenem-resistant Enterobacteriaceae (CRE) clinical isolates in two hospitals in Kuwait and Nigeria.
Methodology: Clinically significant bacterial isolates of patients from Kuwait and Nigeria, identified by VITEK-2 and MALDI-TOF mass spectrometry analysis were studied. Susceptibility testing of selected antibiotics was performed using E-test and broth dilution methods. Genes encoding carbapenemase, β-lactamases, and extended-spectrum β-lactamases (ESBLs) were detected by conventional PCR and sequencing, and whole genome sequencing (WGS) analyses.
Results: Of 400 isolates from Kuwait and Nigeria, 188 (47.0%) and 218 (54.5%) were *Escherichia coli* and 124 (31.0%) and 116 (29.0%) *Klebsiella pneumoniae*, respectively. The prevalence of CRE was 14.0% in Kuwait and 8.0% in Nigeria. The resistance rates of CRE isolates against colistin and tigecycline in Kuwait were 6.6% versus 25.0%, and in Nigeria were 14.2% versus 14.2%, respectively. *bla*$_{OXA-181}$ gene was the commonest in CRE isolates in Kuwait and *bla*$_{NDM-7}$ in Nigeria. The commonest ESBL gene among the CRE isolates was *bla*$_{CTX-M-15}$ in both countries. AmpC resistance genes were present in only Kuwait isolates and mediated by *bla*$_{EBC}$, *bla*$_{CIT}$ and *bla*$_{DHA}$. WGS analysis of 12 selected CRE isolates with carbapenem MICs>32µg/ml but no detectable genes from conventional PCR, revealed the presence of multidrug efflux pump genes such as major facilitator superfamily antibiotic efflux pump and resistance-nodulation-cell division antibiotic efflux pump groups.
Conclusion: The prevalence of CRE was higher among isolates from Kuwait than Nigeria and the genes encoding resistance in CRE were different. The presence of efflux pump was a main mechanism of resistance in most of the Nigerian CRE isolates.

Keywords: CRE; point-prevalence-survey; Kuwait; Nigeria

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Une enquête sur la prévalence ponctuelle des entérobactéries résistantes aux carbapénèmes dans deux villes différentes du Koweït et du Nigeria

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Résumé:

Contexte: La famille des Entérobactéries appartient à l'ordre des Entérobactéries, un grand groupe diversifié de bactéries anaérobies facultatives à Gram négatif qui provoquent parfois des infections multirésistantes dont les options de traitement sont souvent difficiles. Ils sont la principale cause d'infections nosocomiales du sang (BSI) et d'infections des voies urinaires (UTI). L'objectif de l'étude était de mener une enquête sur la prévalence ponctuelle de la résistance aux antimicrobiens et des isolats cliniques d'entérobactéries résistantes aux carbapénèmes (CRE) dans deux hôpitaux au Koweït et au Nigeria.
Méthodologie: Des isolats bactériens cliniquement significatifs de patients du Koweït et du Nigéria, identifiés par analyse par spectrométrie de masse VITEK-2 et MALDI-TOF, ont été étudiés. Les tests de sensibilité des antibiotiques sélectionnés ont été effectués à l'aide des méthodes de test E et de dilution en bouillon. Les gènes codant pour la carbapénémase, les β-lactamases et les β-lactamases à spectre étendu (BLSE) ont été détectés par PCR et séquençage conventionnels et analyses de séquençage du génome entier (WGS).
Résultats: Sur 400 isolats du Koweït et du Nigéria, 188 (47,0%) et 218 (54,5%) étaient *Escherichia coli* et 124 (31,0%) et 116 (29,0%) *Klebsiella pneumoniae*, respectivement. La prévalence de la CRE était de 14,0% au Koweït et de 8,0% au Nigeria. Les taux de résistance des isolats CRE à la colistine et à la tigécycline au Koweït étaient de 6,6% contre 25,0%, et au Nigeria de 14,2% contre 14,2%, respectivement. Le gène *bla*$_{OXA-181}$ était le plus courant dans les isolats CRE au Koweït et *bla*$_{NDM-7}$ au Nigeria. Le gène BLSE le plus courant parmi les isolats CRE était *bla*$_{CTX-M-15}$ dans les deux pays. Les gènes de résistance à l'AmpC étaient présents uniquement dans les isolats du Koweït et médiés par *bla*$_{EBC}$, *bla*$_{CIT}$ et *bla*$_{DHA}$. L'analyse WGS de 12 isolats CRE sélectionnés avec des CMI de carbapénème >32 µg/ml mais aucun gène détectable par PCR conventionnelle, a révélé la présence de gènes de pompe d'efflux multidrogues tels que la pompe d'efflux antibiotique de la superfamille facilitatrice majeure et les groupes de pompe d'efflux antibiotique de division cellulaire de résistance-nodulation.
Conclusion: La prévalence de la CRE était plus élevée parmi les isolats du Koweït que du Nigeria et les gènes codant pour la résistance à la CRE étaient différents. La présence d'une pompe à efflux était un mécanisme principal de résistance dans la plupart des isolats CRE Nigérians.

Mots clés: CRE; enquête de prévalence ponctuelle; Koweït; Nigéria

Introduction:

Species of the family Enterobacteriaceae are members of the normal gut flora that can cause severe healthcare-associated infections such as bloodstream infections, pneumonia, urinary tract infections and intra-abdominal infections. Due to the rise in the proportion of Gram-negative resistant bacteria, for example extended-spectrum β-lactamases (ESBLs)-positive Enterobacteriaceae causing various infections in hospitalized patients, the use of carbapenems in hospital has increased exponentially in the last decade (1). This rise in consumption of carbapenems has been accompanied by the emergence of carbapenem-resistant Enterobacteriaceae (CRE) (2).

The rapid emergence of CRE in this decade has caused tremendous amount of global clinical and public health concerns. According to the Center for Disease Control and Prevention (CDC), CRE is considered as an urgent threat with high mortality and morbidity due to narrow, more toxic, and less effective therapeutic options (3-5). In 2017 for example, there were 13,100 estimated cases of CRE infections among hospitalized patients in the USA with 1,100 estimated deaths and $130 million estimated attributable healthcare costs (5).

The main mechanisms for carbapenem-resistance in Enterobacteriaceae are the production of carbapenemases or ESBLs and/or AmpC cephalosporinase (AmpC) in combination with membrane permeability and efflux pump (6). In Kuwait, the first clinical report of *bla*$_{NDM-1}$–positive *Klebsiella pneumoniae* isolate was detected in 2 patients in Mubarak hospital (7). Then, *bla*$_{VIM-4}$, *bla*$_{OXA-48}$ and *bla*$_{KPC}$ have been discovered from different Enterobacteriaceae such as *Escherichia coli*, *Enterobacter cloacae*, *Morganella morganii*, and *Proteus stuartii* (8-10) in different hospitals in Kuwait. While in Nigeria, the first phenotypic determination of CRE was reported in 2015 (11). Then, this was followed by other reports on molecular characterization of carbapenem resistance genes such as *bla*$_{VIM}$, *bla*$_{GES}$, *bla*$_{NDM}$, *bla*$_{OXA-181}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$ in different Enterobacteriaceae isolates (12-14).

In this study, we conducted point-survey studies on the prevalence of CRE in 2 hospitals in Kuwait and Nigeria, determined their antimicrobial susceptibility pattern and investigated the molecular epidemiological features of the CRE isolates.

Materials and method:

Bacterial isolates

Four hundred consecutive clinically significant non-repetitive isolates from the family Enterobacteriaceae recovered from clinical sam-

ples of patients with proven infections, attending National hospital (NH), Abuja, Nigeria and Mubarak Al Kabeer Hospital, Kuwait, over one-month period (20th January to 27th February, 2019) were studied. NH is a Federal government owned 850-bed teaching hospital with two intensive care units (ICUs), a dialysis unit and an oncology unit, and Mubarak Al Kabeer Hospital is also 850-bed government teaching hospital with two ICUs and a huge dialysis unit. All isolates were sent to the Hospital Infection Reference Laboratory, Faculty of Medicine, Kuwait University, Kuwait for further analysis.

The bacterial isolates were collected from routine laboratory work for patient care in clinical microbiology laboratories of the two hospitals. No additional clinical specimens or clinical data were collected and the isolates were not linked to patient's identity. Ethical approval for the study was therefore not required.

Bacterial identification

Bacterial strains were identified using VITEK-MS system (bioMérieux, Marcy l'Etoile, France), a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. The strains were stored in Brain Heart Infusion Broth (Oxoid Ltd, Basingstoke, Hampshire, England) with 20% glycerol (bio-Mérieux) in -80ºC freezer for further antimicrobial susceptibility and investigation of resistance mechanisms.

Antimicrobial susceptibility testing (AST)

The minimum inhibitory concentrations (MICs) of 13 antimicrobial agents, amikacin, ampicillin, amoxicillin-clavulanic acid, cefepime, cefoxitin, cefotaxime, ceftazidime, ciprofloxacin, colistin, ertapenem, imipenem, meropenem and tigecycline against all isolates were determined using E test (bioMérieux) according to the manufacturer's instruction, except for colistin which AST was performed by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (15).

The following quality control strains were included in each run; *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 (for carbapenem), *E. coli* ATCC 35218 (for β-lactam-β-lactamase inhibitors combination) and colistin-resistant *E. coli,* NCTC 13846 (*mcr-1* positive). The results were interpreted according to the breakpoints and criteria of the CLSI (15). The breakpoints for tigecycline and colistin was based on US Food and Drug Administration (FDA) standards and EUCAST guidelines (16), respectively.

Screening for carbapenemase

All isolates were phenotypically screened for CRE strains using an imipenem-EDTA double disc synergy test and modified Hodge test. These tests were also used to screen and assess the ability of test isolates to produce carbapenemases according to CLSI guidelines (15).

Investigation of resistance mechanisms:

For all CRE isolates, polymerase chain reaction (PCR) was used to detect the genes that encoded the following carbapenemases: *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{OXA-48\ like}$, *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{KPC;}$ and ESBLs: *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CMY}$ (17,18). PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced using a GenAmp PCR system 9700 by cycle sequencing with ABI BigDye® Termination Version 3.1 Kit (Applied Biosystem, Carlsbad, CA, USA). Detection of genes mediating AmpC β-lactamases (*bla*$_{MOX}$, *bla*$_{CIT}$, *bla*$_{DHA}$, *bla*$_{ACC}$, *bla*$_{EBC}$ and *bla*$_{FOX}$) was carried out on CRE isolates cefoxitin-resistant and cefepime susceptible by previously described method (19).

Whole genome sequencing (WGS) and analysis

WGS was carried out on 12 selected isolates with carbapenem MICs > 32 µg/ml in which the carbapenemase genes were undetectable by conventional PCR. These isolates were 5 *M. morganii* (K137, K150, K171, N62 and N85), 4 *P. mirabilis* (K142, N18, N74 and N88), 2 *K. pneumoniae* (K120 and K14) and 1 *E. cloacae* (N40). Bacterial DNA was extracted using QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions. Quantification and quality of the DNA were assessed by Nano-Drop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and analyzed on agarose gel electrophoresis. Purified DNA samples were sent to a commercial company, Novogene Company Limited (Cambridge Science Park, Milton, Cambridge, UK) for WGS. The WGS data (Fastaq) were then uploaded into the web server of the Center for Genomic Epidemiology (http://www.genomic epidemiology.org/).

The plasmid replicon, multi-locus sequence type (MLST), and antimicrobial resistance genes were determined using the following programs; PlasmidFinder (version 2.0.1), MLST (version 2.0.4), and ResFinder (version 4.0), respectively (20-22). The presence of insertion sequences and other mobile genetic elements were determined by ISFinder (https://www-is.biotoul.fr/) to confirm their identity. Multi-drug–resistant (MDR) efflux pumps and outer

membrane porin genes were identified by the Comprehensive Antibiotic Resistant Database (CARD) analysis.

Results:

Distribution of the isolates:

Table 1 shows the distribution of the clinical isolates from Kuwait and Nigeria. The top 3 of the 200 Enterobacteriaceae isolates from Kuwait were *E. coli* 94 (47%); *Klebsiella pneumoniae* 62 (31%) and *Serratia marcescens* 11 (5.5%). Others were *Proteus mirabilis* 8 (4%); *Morganella morganii* 7 (3.5%); *Citrobacter freundii* 7 (3.5%); *Enterobacter cloacae* 6 (3%); *Raoultella planticola* 3 (1.5%) and *Providentia stuartii* 2 (1%). The top 3 isolates from Nigeria were *E. coli* 109 (54.5%); *K. pneumoniae* 58 (29%) and *P. mirabilis* 19 (9.5%). Other isolates were *E. cloacae* 5 (2.5%); *M. morganii* 3 (1.5%); *C. freundii* 3 (1.5%); *S. marcescens* 2 (1%) and *Aeromonas hydrophila* 1 (0.5%).

A total of 28 (14%) isolates from Kuwait were CRE. These were 6 (21.4%) *P. mirabilis* 5 (17.9%); *M. morganii* 4 (12.3%); *K. pneumoniae* 5 (17.9%); *E. coli* 2 (7.1%); *S. marcescens* 2 (7.1%); *C. freundii* 2 (7.1%); *P. stuartii* 1 (3.6%); *K. aerogenes* and *E. cloacae* 1 (3.6%). Their sources were 7 (25%) urinary tract infections (UTIs); 6 (21.4%) lower respiratory tract infections (LRTIs), 4 (14.3%) blood stream infections (BSIs), 3 (10.7%) wound infections, 4 (14.3%) tissues, 2 (7.1%) pus aspirates, 1 (3.6%) peritoneal fluid, and 1 (3.6%) bed sore. By contrast, only 16 (8%) of the Nigerian isolates were CRE; 3 (18.8%) *K. pneumoniae*, 2 (12.5%) *E. coli*, 2 (12.5%) *E. cloacae*, 6 (37.5%%) *P. mirabilis* and 3 (18.8%) *M. morganii*. They were isolated from 9 (56.3%) wound infections, 3 (18.8%) UTIs, 1 (6.3%) BSIs, 1 (6.3%) cerebrospinal fluid, 1 (6.3%) ventricular shunt and 1 (6.3%) from eye swab.

Table 1: Distribution of clinical bacterial isolates from two hospitals in Kuwait and Nigeria

Bacterial isolate	**Number (%) of isolates**	
	Kuwait	**Nigeria**
Escherichia coli	94 (47)	109 (54.5)
Klebsiella pneumoniae	62 (31)	58 (29)
Serratia marcescens	11 (5.5)	2 (1)
Proteus mirabilis	8 (4)	19 (9.5)
Morganella morganii	7 (3.5)	3 (1.5)
Citrobacter freundii	7 (3.5)	3 (1.5)
Enterobacter cloacae	6 (3)	5 (2.5)
Raoultella planticola	3 (1.5)	0 (0)
Providentia stuartii	2 (1)	0 (0)
Aeromonas hydrophila	0	1 (0.5)
Total	**200 (100.0)**	**200 (100.0)**

Table 2: Antimicrobial susceptibility profiles of clinical Enterobacteriaceae isolates from Kuwait and Nigeria

Antimicrobial (breakpoints in µg/ml)	**Kuwait (n=200)**				**Nigeria (n=200)**			
	MIC range	**MIC_{50}**	**MIC_{90}**	**% resistance**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**% resistance**
Amikacin (16)	1 - >256	4	12	5	0.25 - >256	3	8	3
Ampicillin (8)	0.5 - >256	256	256	93	0.25 - >256	>256	>256	89
Amoxicillin-clavulanic acid (8)	0.38 - >256	6	32	31	0.38 - >256	6	32	34
Cefepime (2)	0.016 - >256	1	>256	47	0.016 - >256	6	>256	57
Cefoxitin (8)	1 - >256	3	256	29	0.75 - >256	4	>256	27
Cefotaxime (1)	0.012 - >256	256	256	59	0.006 - >256	>256	>256	59
Ceftazidime (4)	0.047 - >256	3	256	48	0.016 - >256	3	>256	47
Ciprofloxacin (1)	0.004 - >32	0.5	32	43	0.004 - >32	8	>32	58
Colistin (2)*	0.125 - >256	0.75	>256	1.7	0.094 - >256	0.5	>256	2.3
Ertapenem (0.5)	0.008 - >32	0.094	0.5	7	0.002 - 3	0.032	0.25	1
Imipenem (1)	0.094 - >32	0.25	2	13	0.032 - 12	0.25	1	8
Meropenem (1)	0.064 - >32	0.125	0.25	3	0.012 - 6	0.094	0.19	1
Tigecycline* (2)	0.125 - >256	1	4	9.8	0.094 - >256	0.5	3	4.5

*Excluding the following organisms from the calculation: *Proteus mirabilis*, *Morganella morganii*, *Providentia stuartii* and *Serratia marcescens*
MIC=minimum inhibitory concentration; n=number

Antimicrobial susceptibility test results:

Antimicrobial susceptibility results including MIC range, MIC_{50}, MIC_{90} and percentage of resistance for all the 400 isolates from Kuwait and Nigeria are shown in Table 2. Among the isolates from Kuwait, colistin, meropenem, amikacin, ertapenem and tigecycline demonstrated excellent activities with low resistance rates of 1.7%, 3%, 5%, 7%, 9.8%, respectively. Similarly, meropenem, ertapenem, colistin, amikacin, tigecycline and imipenem exhibited excellent activities on the isolates from Nigeria with low resistance rates of 1%, 1%, 2.3%, 3%, 4.5% and 8%, respectively. The majority of the isolates from Kuwait (93%) and Nigeria (89%) were resistant to ampicillin.

The resistance rates to cefepime, cefotaxime and ceftazidime were relatively high in both Kuwait (47%, 59%, and 48%, respectively) and Nigeria (57%, 59% and 47%, respectively). Ciprofloxacin also demonstrated very poor activities against isolates from Kuwait and Nigeria with resistance rates of 43% and 58%, respectively.

Antimicrobial susceptibility of the CRE isolates:

Antimicrobial susceptibility rates of the CRE isolates are shown in Table 3. The most active antimicrobial agents against CRE isolates from Kuwait were colistin to which only 6.6% were resistant, followed by meropenem (17. 8%), tigecycline (25%) and amikacin (28%). The most active antibiotics against the Nigerian isolates were amikacin (0% resistance), ertapenem and meropenem (12.5% each), colistin (14.2%) and tigecycline (14.2%). The majority of the isolates in Kuwait and Nigeria showed high resistance to amoxicillin-clavulanic acid with rates of 75% and 68.7%, respectively.

Resistance to other β-lactam antibiotics in Kuwaiti and Nigerian CRE isolates were relatively high; respective resistance rates to cefepime were 42.8% and 56.2%, ceftazidime 39.2% and 37.5%, cefotaxime 57.1% and 56.2% and cefoxitin 64.2% and 62.5%. Half (50%) of the isolates from Kuwaiti were resistant to ciprofloxacin compared to 31% from Nigeria.

Table 3: Antimicrobial susceptibility of clinical carbapenem-resistant Enterobacteriaceae isolates from Kuwait and Nigeria

Antimicrobial (breakpoints in µg/ml)	**Kuwait (n=28)**				**Nigeria (n=16)**			
	MIC range	**MIC_{50}**	**MIC_{90}**	**% resistance**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**% resistance**
Amikacin (16)	0.5 - >256	3	24	28	1.5 - 6	2	3	0
Ampicillin (8)	1.5 - >256	>256	>256	96.4	0.5 - >256	>256	>256	87.5
Amoxicillin-clavulanic acid (8)	0.38 - >256	24	>256	75	0.5 - >256	24	>256	68.7
Cefepime (2)	0.032 - >256	0.38	>256	42.8	0.094 - 48	3	32	56.2
Cefoxitin (8)	2 - >256	24	>256	64.2	2 - >256	16	>256	62.5
Cefotaxime (1)	0.012 - >256	8	>256	57.1	0.012 - >256	>256	>256	56.2
Ceftazidime (4)	0.047 - >256	0.5	>256	39.2	0.047 - >256	0.19	>256	37.5
Ciprofloxacin (1)	0.008 - >32	1	>32	50	0.016 - >32	0.25	16	31.2
Colistin* (2)	0.38 - 32	32	32	6.6	0.13 - 8	8	8	14.2
Ertapenem (0.5)	0.016 - >32	0.38	>32	46.4	0.012 - 3	0.032	3	12.5
Imipenem (1)	0.19 - >32	3	>32	89.2	1.5 - 12	2	8	100
Meropenem (1)	0.125 - >32	0.25	4	17.8	0.064 - 6	0.19	4	12.5
Tigecycline* (2)	0.25 - >256	12	12	25	0.094 - 3	3	3	14.2

*Excluding the following organisms from the calculation: *Proteus mirabilis*, *Morganella morganii*, *Providentia stuartii*, and *Serratia marcescens*
MIC=minimum inhibitory concentration; n=number

Table 4: Carbapenemase resistant genes harbored by CRE strains in Kuwait and Nigeria

Detected genes	**Number of CRE isolates harboring specific genes**					
	Kuwait (n=10)					**Nigeria (n=2)***
	K. pneumoniae	***E. coli***	***P. mirabilis***	***M. morganii***	***S. marcescens***	***E. coli***
$bla_{OXA-181}$	1	2	0	1	0	0
$bla_{OXA-833}$	1	0	0	0	0	0
bla_{OXA-48}	0	0	1	0	0	0
$bla_{OXA-244}$	0	1	0	0	0	0
$bla_{OXA-233}$	0	0	0	0	0	0
bla_{KPC-2}	0	0	0	0	1	0
bla_{NDM-7}	0	0	0	0	0	2
$bla_{OXA-232}$ + bla_{NDM-5}	2	0	0	0	0	0
Total	**4**	**3**	**1**	**1**	**1**	**2**

*Only *E. coli* harbored a carbapenemase gene among the Nigerian isolates

Carbapenemase genes

A total of 10 (35.7%) out of 28 CRE isolates from Kuwait harbored 1 or 2 genes mediating carbapenemase production as shown in Table 4. Four (40%) of the 10 isolates harbored *bla*$_{OXA-181}$. Other genes detected were *bla*$_{OXA-48}$, *bla*$_{OXA-244}$, *bla*$_{OXA-833}$, and *bla*$_{KPC-2}$. Two *K. pneumoniae* isolates co-harbored both *bla*$_{OXA-232}$ and *bla*$_{NDM-5}$. Only 2 (12.5%) of the 16 Nigerian CRE isolates harbored carbapenemase gene, *bla*$_{NDM-7}$.

Other mechanisms associated with carbapenem resistance:

The conventional PCR failed to detect the usual genes mediating carbapenemases production in 18 (64.2%) out of the 28 CRE isolates from Kuwait and 14 (87.5%) of the 16 isolates from Nigeria. From Kuwait, these isolates were; 5 *M. morganii*, 5 *P. mirabilis*, 2 *E. coli*, 2 *P. stuartii*, 1 *S. marcescens*, 1 *E. cloacae*, 1 *K. aerogenes* and 1 C. *freundii*. Those from Nigeria included 6 *P. mirabilis*, 3 *K. pneumoniae*, 3 *M. morganii* and 2 *E. cloacae*.

However, ESBL encoding genes (*bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM)}$ and *bla*$_{CMY}$ mediating AmpC were detected by conventional PCR in 24 and 14 isolates, respectively as shown in Tables 5 and 6. Among the ESBL-positive isolates, *bla*$_{CTX-M-15}$ were detected in 6 isolates from Kuwait, *bla*$_{CTX-M-65}$ in 2 and *bla*$_{CTX-M-14}$ in 1, and *bla*$_{SHV-1}$ gene was detected in 4 isolates. Four isolates from Nigeria carried *bla*$_{CTX-M-15}$ and 3 *bla*$_{CTX-M-3}$. None of the Nigerian isolates harbored any AmpC gene.

Results of WGS analysis:

As shown in Table 7, there were several antimicrobial resistance genes that mediated resistance to several antimicrobial agents among the randomly selected 12 non-carbapenemase-producing (NCP) CRE isolates. These genes were located either on chromosome or plasmid. All 12 NCP CRE isolates harbored homologues of *KpnE*, *KpnF*, *KpnG*, or *KpnH* genes alone or in combinations, which were associated with major facilitator superfamily (MFS) antibiotic efflux pump and its regulations (CRP gene).

Five *M. morganii* NCP CRE isolates (K137, K150, K171, N62 and N85) harbored AmpC β-lactamase gene, *bla*$_{DHA}$. Six NCP CRE isolates (K142 *P. mirabilis*, K150 *M. morganii*, N18 *P. mirabilis*, N62 *M*. *morganii*, N85 *M*. *morganii* and N88 *P. mirabilis*) harbored *qacEdelta1* genes responsible for mediating the major facilitator superfamily (MFS) antibiotic efflux pump.

All, except 2 (K120 *K. pneumoniae* and K14 *K. pneumoniae*) isolates, harbored *rsmA* genes that conferred resistance-nodulation-cell division (RND) antibiotic efflux pump. One N40 *E. cloacae* isolate carried an efflux pump with reduced permeability *marA* gene in addition with 2 AmpC β-lactamase genes, *bla*$_{AmpH}$ and *bla*$_{ACT-29}$. Carbapenemase genes of the *bla*$_{OXA-232}$ and *bla*$_{NDM-5}$ varieties were detected in one isolate (K14 *K. pneumoniae*) as well as AmpC β-lactamase enzyme, *bla*$_{AmpH}$ and other 3 β-lactamase genes (*bla*$_{CTX-M-15}$, *bla*$_{SHV-1}$ and *bla*$_{TEM-1)}$.

MLST results of the 12 CRE isolates:

Of the 12 CRE isolates, only 2 were evaluable for MLST on the Plasmid Finder. These were *K. pneumoniae* strains K120 and K14, both of which belonged to sequence type ST231. The remaining isolates consisting of 5 *M. morganii*, 4 *P. mirabilis* and 1 *E. cloacae* were not evaluated as *M. morganii* and *Proteus* spp. had no allelic profile developed at the time of our investigation for current DNA-based typing assay.

Analysis of mobile genetic elements (MGEs):

Plasmid Finder showed that K120 *K. pneumoniae* isolate belonged to sequence type ST231 and harbored 6 plasmids; Col(pHAD28), Col440I, IncFIA, IncFIB(pQil), IncFII(K) and IncFII(pAMA1167-NDM-5). In addition, the 115300 bp DNA IncFIB (pQil) plasmid carried *bla*$_{ampH,}$ *bla*$_{SHV-1}$ and *bla*$_{TEM-1}$ genes that mediated β-lactam resistance. Insertion sequences IS26, ISKpn7, ISKpn6 and Tn3 transposase, detected through IS Finder, were also present.

The other sequence type ST231 K14 *K. pneumoniae* isolate carried 7 replicon plasmid types; Col(pHAD28), Col440I, ColKP3, IncFIB (pKPHS1), IncFIB(pQil), IncFII and IncFII(K). ColKP3 plasmid encoded for carbapenemase genes, *bla*$_{OXA-232}$ and *bla*$_{NDM-5}$. Plasmid IncFII harbored *bla*$_{ampH,}$ *bla*$_{CTX-M-15,}$ *bla*$_{SHV-1,}$ and *bla*$_{TEM-1}$ genes. These isolates also carried Insertion sequences IS*Ecp1*IS26, ISKpn7, ISKpn6 and Tn3 transposase.

Table 5: β-lactamase and ESBL resistant genes harbored by CRE isolates in Kuwait and Nigeria

Detected genes	**Number of CRE isolates harboring specific genes**							
	Kuwait (n=12)			**Nigeria (n=12)**				
	K. pneumoniae	***E. coli***	***P. mirabilis***	***K. pneumoniae***	***E. coli***	***P. mirabilis***	***M. morganii***	***E. cloacae***
bla$_{CTX-M-15}$	1	0	0	0	0	0	1	0
bla$_{CTX-M-14}$	0	1	0	0	0	0	0	0
bla$_{CTX-M-65}$	1	0	1	0	0	0	0	0
bla$_{CTX-M-3}$	0	0	0	0	0	0	2	0
bla$_{SHV-1}$	2	0	0	0	0	0	0	0
bla$_{TEM-1}$	0	0	0	0	2	3	0	0
bla$_{CTX-M-15}$ + *bla*$_{SHV-1}$ + *bla*$_{TEM-1}$	2	0	0	0	0	0	0	0
bla$_{CTX-M-15}$ + *bla*$_{CMY-42}$	0	1	0	0	0	0	0	0
bla$_{CTX-M-15}$ + *bla*$_{CMY-166}$	0	1	0	0	0	0	0	0
bla$_{CTX-M-15}$ + *bla*$_{TEM-1}$	1	1	0	1	0	0	0	1
bla$_{CTX-M-3}$ + *bla*$_{TEM-1}$	0	0	0	1	0	0	0	0
bla$_{CTX-M-15}$ + *bla*$_{SHV-37}$ + *bla*$_{TEM-1}$	0	0	0	1	0	0	0	0
Total	**7**	**4**	**1**	**3**	**2**	**3**	**3**	**1**

CRE = Carbapenem-resistant Enterobacteriaceae; ESBL = extended-spectrum β-lactamase

Table 6: AmpC cephalosporinase genes among cefoxitin-resistant and cefepime-susceptible CRE in Kuwait

Detected genes	**Number of CRE isolates harboring specific genes in Kuwait (n=14)**						**Total**
	E. cloacae	***E. coli***	***K. pneumoniae***	***K. aerogenes***	***C. freundii***	***M. morganii***	
bla$_{MOX}$	0	0	0	0	0	0	0
bla$_{CIT}$	0	2	0	0	1	0	3
bla$_{DHA}$	0	0	2	0	0	1	3
bla$_{ACC}$	0	0	0	0	0	0	0
bla$_{EBC}$	4	0	1	3	0	0	8
bla$_{FOX}$	0	0	0	0	0	0	0
Total	**4**	**2**	**3**	**3**	**1**	**1**	**14**

Table 7: Resistance mechanisms of the carbapenemase-negative Enterobacteriaceae isolates to different antimicrobial agents detected by whole genome sequencing

Designated ID number	**Bacterial isolate**	**β-lactam-encoding resistance genes**	**Non-β-lactam-encoding resistance genes**	**Other resistance genes**	**Antibiotic target**	**Gene location**
K120	*K. pneumoniae*	*bla*$_{ampH}$, *bla*$_{SHV-1}$, *bla*$_{TEM-1}$	*AAC(6')-Ib, aadA2, mphA, catI, arr-2, PBP3,* gyrA, *EF-Tu, parC, folP, UhpT, FosA6, QnrS1, dfrA12, sul1, ErmB*	Efflux pump: *marR, oqxA, baeR, H-NS, CRP, adeF, KpnE, KpnF, KpnG, KpnH, emrR, msbA, marA*	Aminoglycoside, macrolide, phenicol, rifamycin, β-lactam, fluoroquinolone, elfamycin, fluoroquinolone, sulfonamides, fosfomycin, quinolone, trimethoprim resistant dihydrofolate reductase dfr, sulfonamide, Erm 23S ribosomal RNA methyltransferase.	Plasmid IncFIB(pQil)
K14	*K. pneumoniae*	*bla*$_{ampH}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-232}$, *bla*$_{NDM-5}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-1}$, *bla*$_{TEM-1}$	*AAC(6')-Ib, aadA2, mphA, catI, arr-2, PBP3,* gyrB, *EF-Tu, parC, folP, UhpT,*	Efflux pump: *marR, oqxA, baeR, H-NS, CRP, FosA6, adeF, KpnE, KpnF, KpnG, KpnH, emrR, msbA, marA*	Aminoglycoside, macrolide, phenicol, rifamycin, β-lactam, fluoroquinolone, elfamycin, fluoroquinolone, sulfonamides, fosfomycin.	Plasmid IncFIB(pQil), ColKP3, IncFII

K137	*M. morganii*	*bla*$_{DHA-17,}$	*CatIII, PBP3,* gyrB, *EF-Tu*	Efflux pump: *rsmA, CRP, KpnH*	CAT, β-lactam, fluoroquinolone, elfamycin.	Chromosome
K142	*P. mirabilis*		*catA4, catII, sul1, sul2, ANT(3'')-IIa, aadA2, SAT-2, dfrA1, PBP3,* gyrB, *EF-Tu*	Efflux pump: *KpnF*, *adeF*, *CRP, tet(D), qacEdelta1, rsmA, KpnH*	CAT, sulfonamide, aminoglycoside, streptothricin acetyltransferase (SAT), trimethoprim resistant dihydrofolate reductase dfr, β-lactam, fluoroquinolone, elfamycin.	Chromosome
K150	*M. morganii*	*bla*$_{DHA-17}$	*CatIII, mphA, sul1, dfrA24, cmlA6, aadA, ANT(2'')-Ia, PBP3,* gyrB, *EF-Tu, tetR*	Efflux pump: *CRP, KpnH, rsmA, qacEdelta1, tet(B)*	CAT, macrolide phosphotransferase (MPH), Sulfonamide, trimethoprim resistant dihydrofolate reductase dfr, phenicol, aminoglycoside, β-lactam, fluoroquinolone, elfamycin, tetracycline	Chromosome
K171	*M. morganii*	*bla*$_{DHA-13}$	*PBP3,* gyrB, *EF-Tu,*	Efflux pump: *rsmA, tet(D), CRP, KpnH*	β-lactam, fluoroquinolone, elfamycin.	Chromosome
N18	*P. mirabilis*	*bla*$_{TEM-1}$	*APH(6)-Id, catA4, sul1, sul2, QnrD2, PBP3,* gyrB, *EF-Tu,*	Efflux pump: *KpnF*, *adeF*, *CRP, tet(D), qacEdelta1, rsmA, KpnH*	Aminoglycoside, CAT, sulfonamide, quinolone resistance protein (qnr), β-lactam, fluoroquinolone, elfamycin	Chromosome
N40	*E. cloacae*	*bla*$_{ACT-29,}$ *bla*$_{ampH,}$	*FosA2, PBP3, EF-Tu*	Efflux pump: *KpnF*, *adeF*, *CRP, rsmA, KpnE, marA*, ramA, H-NS, *adeF*, *oqx*A, msbA, baeR, emrB, emrR, marR	Fosfomycin, β-lactam, elfamycin,	Chromosome
N62	*M. morganii*	*bla*$_{DHA-1,}$ *bla*$_{CTX-M-15,}$ *bla*$_{OXA-1}$	*CatII, AAC(3)-IIe, APH(6)-Id, sul1, sul2, dfrA1, AAC(6')-Ib-cr, dfrA14, PBP3,* gyrB, EF-Tu	Efflux pump: qacEdelta1, KpnH, CRP, rsmA, *tet(D),*	Phenicol, aminoglycoside, sulfonamide, diaminopyrimidine, fluoroquinolone, aminoglycoside, tetracycline, trimethoprim, β-lactam, fluoroquinolone, elfamycin.	Chromosome
N74	*P. mirabilis*		*CatIII, FosA2, catA4, gyrB, PBP3, EF-Tu, catA4, AAC(2')-Ia*	Efflux pump: KpnH, KpnF, CRP, rsmA, *tet(B), adeF, CRP, tetR, tet(D)*	Chloramphenicol, fosfomycin, chloramphenicol, fluoroquinolone, β-lactam, elfamycin, CAT, aminoglycoside.	Chromosome
N85	*M. morganii*	*bla*$_{DHA-1,}$ *bla*$_{CTX-M-15,}$ *bla*$_{OXA-1}$	*CatII, AAC(3)-IIe, APH(6)-Id, sul1, sul2, dfrA1, AAC(6')-Ib-cr, dfrA14, PBP3,* gyrB, EF-Tu	Efflux pump: qacEdelta1, KpnH, CRP, rsmA, *tet(D)*	Phenicol, aminoglycoside, sulfonamide, diaminopyrimidine, fluoroquinolone, aminoglycoside, tetracycline, trimethoprim, β-lactam, fluoroquinolone, elfamycin.	Chromosome
N88	*P. mirabilis*	*bla*$_{TEM-1}$	*APH(6)-Id, catA4, sul1, sul2, QnrD2, PBP3,* gyrB, *EF-Tu,*	Efflux pump: *KpnF, KpnH adeF, CRP, tet(D), qacEdelta1, rsmA*	Aminoglycoside, CAT, sulfonamide, quinolone resistance protein (qnr), β-lactam, fluoroquinolone, elfamycin.	Chromosome

CAT = chloramphenicol acetyltransferase

Discussion:

Infected patients with CRE have higher mortality and morbidity rates than those infected by susceptible Enterobacteriaceae strains and therefore, require proper and effective antimicrobial therapy. During a one-month point survey, only 14% and 8% of the Enterobacteriaceae isolates were CRE in Kuwait and Nigeria, respectively. The production of carbapenemases mediated by $bla_{\text{OXA-181}}$ gene was a major mechanism of resistance among the CRE isolates in Kuwait. However, carbapenemase production was not a major mechanism of resistance in the CRE from the Nigerian center.

Our data demonstrated that the type of resistance genes harbored by clinical isolates of CRE reported previously from Kuwait differed from our present finding of predominance of OXA-181 carbapenemase (9,10). It is possible that the other genes are being replaced systematically by $bla_{\text{OXA-181}}$, perhaps via horizontal transfer by plasmid encoded resistance factors, in our hospitals. Our present finding was also discordant with some recent surveillance reports emanating from China (23) and several European countries, including Turkey and Israel (24). In the European survey involving 455 sentinel hospitals in 36 countries, 37% of *K. pneumoniae* and 19% of *E. coli* were carbapenemase-producers including KPC, NDM, OXA-48-like, or VIM (24), while in China, NDM and KPC-2 were responsible for 90% of CRE (23). Although the number of CRE reported among the Nigerian isolates were half of those from Kuwait, the carbapenemases produced by CRE from both centers were completely different. In Nigeria, only 2 CRE isolates produced NDM-7 carbapenemase which had never been encountered before among clinical isolates in Kuwait.

In our current survey, 67.8% and 87.5% of CRE isolates were negative for the carbapenemases investigated by conventional PCR assays among the Kuwait and Nigerian isolates, respectively. This large number of NCP CRE in Nigeria is unprecedented in the literature. For instance, in the European survey conducted by Grundmann et al., (24) in 2017, 29% of *K. pneumoniae* and 60% of *E. coli* isolates resistant to carbapenem were negative for KPC, NDM, OXA-48-like, or VIM carbapenemase production. The reason for this observation in both our study and the European study may be due to the production of other non-tested carbapenemase or reduced permeability. For this reason, we randomly selected 12 of such isolates (with MIC ≥ 32µg/ml) for WGS.

The WGS analysis revealed that the mechanism of resistance to the carbapenems in the 12 isolates was mainly due to the presence of multidrug efflux pump (MDEP) genes of the MFS antibiotic efflux pump and resistance-nodulation-cell division (RND) antibiotic efflux pump groups. In addition, one isolate from Kuwait harbored 2 carbapenemase genes, $bla_{\text{OXA-232}}$ and $bla_{\text{NDM-5}}$, that were undetectable by PCR. It is conceivable that these genes were missed because of lack of inclusion of adequate specific primers in the PCR assays. The fact that many of our CRE isolates from both countries did not produce carbapenemases is not new as previous similar studies have demonstrated low- and high-level carbapenem resistance in *Klebsiella* species and *Enterobacter* species without carbapenemase production. In these isolates, resistance was mainly due to combination of production of β-lactamase and impermeability caused by loss of outer membrane proteins (OMPs) (25,26) similar to our findings, ably demonstrated by WGS.

In addition, efflux pump and AmpC overexpression or loss of porins have been described as features of some NCP CRE isolates (26,27) as were the case with our isolates. In a recent report from Nigeria by Akinpelu et al., (28) in 2020, 18 NCP CRE *Klebsiella* spp. isolated from clinical samples were positive for the presence of efflux pump activity tested via ethidium bromide cartwheel method and biofilm forming ability via tissue culture, thereby corroborating our findings on the Nigerian isolates. All the 4 *P. mirabilis* and 5 *M. morganii* isolates also harbored chromosomally encoded β-lactam-encoding resistance gene. However, there were 2 replicon plasmid types (Col3M) detected in N74 *P. mirabilis* with 2 different identities (97.78 and 96.3) and 2 different positions in the contig but with hardly any significant resistance by itself.

Alarmingly, many of the CRE isolates from Kuwait and Nigeria were resistant to the last line antibiotics for the treatment of CRE or MDR infections. Colistin and tigecycline demonstrated relatively unacceptable poor levels of activities against these isolates. This observation may reflect the massive abuse of these antimicrobial agents in our hospitals, particularly in the intensive care unit patients, thereby creating immense selection pressure. However, the resistance level encountered against colistin in our study was lower than the 28.3% resistance rate reported in the European surveillance study (24), but higher than the 1.1–6.2% reported in China (29). The resistance to tigecycline in our study was much higher than

the 5.2% resistance rate reported in the European surveillance study (24).

Analysis of the ESBLs showed that *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-14}$ were the most commonly identified genes in our isolates. These ESBL gene types have been widely reported worldwide as the genes that encode CTX-M enzymes (*bla*$_{CTX-M}$) which can be horizontally mobilized by different genetic elements (30). Detection of AmpC genes was investigated only in the CRE isolates that were cefoxitin-resistant and cefepime-susceptible, and found only in 14 CRE isolates from Kuwait and none in the Nigerian CRE. The most common genes were *bla*$_{FBC}$, *bla*$_{CIT}$, and *bla*$_{DHA}$. AmpC overexpression phenotype had been demonstrated in a previous report from Switzerland to be a feature of carbapenem-resistant *E. cloacae* (26). This is in keeping with recent reports on acquisition of plasmid-mediated cephalosporinase producing Enterobacteriaceae after a travel to the tropics like Asia, Latin America and Africa (31).

Our study is limited by the fact that only one center per country were included, other mechanisms of carbapenem resistance in all isolates such as permeability problem or porin loss were not investigated, the number of isolates investigated by WGS were relatively small and susceptibility against the new antibiotic combinations such as ceftazidime-avibactam, meropenem-vaborbactam, imipenem-relebactam and ceftolozane-tazobactam were not done; the latter being due to unavailability of these antibiotics in our countries at the time of the study.

In conclusion, the study has revealed that the prevalence of CRE in Kuwait was much higher than in Nigeria, that the resistant isolates were mediated by diverse mechanisms including production of OXA-181, NDM, presence of efflux pumps, combination of ESBLs and AmpC enzymes. It is advocated that preventing CRE infections and containing the spread of carbapenem resistance should be front runners in the infection control guidelines for each hospital.

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Contributions of authors:

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