ORIGINAL ARTICLE

Preliminary Screening of *Bla*$_{NDM-1}$ Gene of Carbapenem-resistant *Klebsiella pneumoniae* in Clinical Samples of Patients at a Teaching Hospital

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ABSTRACT

Introduction: The emergence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is due to presence of the *bla*$_{NDM-1}$ gene that expresses the NDM-1 enzyme. As antibiotic resistance is a threat to public health, it is important to determine the prevalence of CRKP isolated from patients at a teaching hospital in the east coast of Peninsular Malaysia and understand the demographical trend of the bacteria in patients to keep infection at bay. **Methods:** A total of 213 *Klebsiella pneumoniae* isolates from the year 2017 to 2019, of non-repetitive clinical specimens of patients were tested for sensitivity against carbapenems using the disc-diffusion method. Phenotypic detection of carbapenemase was performed using the modified Hodge test and polymerase chain reaction was used to detect the presence of *bla*$_{NDM-1}$ from bacterial DNA. **Results:** Isolates with *bla*$_{NDM-1}$ gene was present in 93.4% (n=199) of 213 samples, with the highest number in blood (20.7%, n=44) and urine samples (20.7%, n=44). CRKP isolates producing NDM-1 enzyme were found to be the highest in male patients (56.8%, n=113) and in those aged between 61 and 80 years (49.7%, n=99), although it was not significantly associated. **Conclusion:** This study identified the presence of *bla*$_{NDM-1}$ in a large proportion of CRKP isolates obtained from samples at the teaching hospital. This may require the formulation of protocols to screen for CRKP and reduce the risk of infection, especially among elderly males. The Ministry of Health may need to implement screening protocols to reduce infection risk.

Keywords: Carbapenem-resistant Enterobacteriaceae; Carbapenemase; *Klebsiella pneumoniae*; *bla*$_{NDM-1}$

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INTRODUCTION

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a notorious multidrug-resistant pathogen commonly acquired in a hospital setting. Its resistance is due to the production of carbapenemase enzymes like metallo beta-lactamase, which allows the bacteria to hydrolyse the β-lactam ring in antibiotics except aztreonam (1). Carbapenems belong to the β-lactamase class of antibiotics, and its action is similar to penicillin, which inhibits the synthesis of bacterial cell wall. However, it has a broader spectrum of antibacterial activity and is quite effective in the treatment of multi-drug resistant bacteria. However, over the past decades, resistance to this vital antibiotic is emerging at a rapid rate (2). As more CRKP is isolated in the hospital setting and from the community, research focus is now concentrated on controlling its infection. Hospital protocols on CRKP detection is therefore very important in treating and preventing the increase of resistance currently encountered (3).

Our previous review reported that the prevalence of CRKP in several hospitals in Malaysia was increasing and the most commonly found carbapenemase gene in CRKP was *bla*$_{NDM-1}$ (4). For example, Zainol et al. (5) reported the prevalence of positive NDM-1 gene was 29% (n=18), and 89% (n=16) were *Klebsiella pneumoniae*. Zaidah et al. (6) reported 95% (n=388) of the isolated Carbapenem resistant Enterobacteriaceae (CRE) in their study was *Klebsiella pneumoniae* and 82% (n=112) showed positive detection of NDM-1 gene. Hence, the aim

of this study was to investigate the prevalence of *bla*$_{NDM-1}$ positive CRKP isolated from different clinical specimens and associate it with the sociodemographic data of patients at a teaching hospital in Kelantan, a northeastern state in Peninsular Malaysia, within the recent three years. As part of the medical faculty of a renowned public university, the teaching hospital (located at a suburban area in the state capital of Kota Bharu) is one of the primary referral hubs for medical cases in the east coast of Peninsular Malaysia. This study has updated the previous knowledge of CRKP that reported very low prevalence of CRKP in Malaysia and the need for more research in this area toward understanding the underlying mechanism for carbapenem resistance because carbapenems are considered as the last option when treating infections due to multidrug-resistant microorganisms in hospital settings.

MATERIALS AND METHODS

Samples and isolates

This retrospective descriptive study involved the retrieval of CRKP strains collected in the stock culture laboratory at the Department of Medical Microbiology and Parasitology of the hospital. All patients' data pertaining to the organisms isolated was retrieved from the hospital Laboratory Information System (LIS) databases available in the department and ethical approval was obtained from Human Research Ethics Committee, Universiti Sains Malaysia (reference no: USM/JEPeM/19110715). Non-repetitive CRKP isolates from blood, urine, sputum, endotracheal intubation, tracheal aspirates and other samples collected between 2017 and 2019 were retrieved and identified based on growth characteristics before being subjected to basic biochemical testing and confirmed using the Vitek GNI card in the Vitek2 automated identification system (bioMe´rieux Vitek, Durham, North Carolina, USA).

Antibiotic susceptibility tests

The susceptibility of isolates against meropenem, imipenem and ertapenem were tested using the disc-diffusion method. The interpretation of sensitivity results were referred to the Clinical and Laboratory Standards Institute (CLSI) criteria for carbapenems (7). The disc zones of inhibition ≤19 mm indicated resistance; 20 to 22 mm indicated intermediate resistance; and ≥23 mm indicated sensitivity. Bacterial colonies were considered as suspected carbapenemase producers when showed a zone of inhibition ≤18 mm in diameter for ertapenem and ≤19 mm for meropenem/imipenem. Colonies that demonstrated reduced susceptibility towards ertapenem, imipenem or meropenem were further tested for minimal inhibitory concentration (MIC) using the E-test method.

Detection of carbapenemase

The modified Hodge test (MHT) was used for phenotypic detection of carbapenemase in bacterial colonies. The MHT was performed according to the CLSI guidelines (8). *Klebsiella pneumoniae* (positive control strain: ATCC BAA-1705; negative control strain: ATCC BAA-1706) was resuspended in 0.5 ml MacFarland's suspension and was diluted with sterile saline at a ratio of 1 to 10. The diluted bacterial suspension was inoculated on Mueller Hinton agar and left to dry for five minutes. An imipenem disc (10 µg) was then placed on the centre of the agar. A few colonies of *Klebsiella pneumoniae* were picked and streaked in a straight line, from the edge of the disc up to a distance of at least 20 mm. The plates were incubated at 37^{0}C for 24 hours and examined. Inoculated plates were checked for enhanced growth around the intersection of the streak (clover-leaf indentation) (9).

Detection of *bla*$_{NDM-1}$ gene

Boiling method was used for the genomic deoxyribonucleic acid (DNA) extraction as described by Oliveira et al. (10). Two fresh colonies of *Klebsiella pneumoniae* were mixed and suspended in 300 µL distilled water. The suspension was heated at 100^{0}C for 10 minutes. After cooling down to room temperature, the suspension was centrifuged at 15,000 g for five minutes and the supernatant was used as a source of DNA template. Polymerase Chain Reaction (PCR) amplification was carried out on the extracted DNA to detect the *bla*$_{NDM-1}$ gene using published primers stated in Table I (11).

All amplifications of PCR were conducted using the exTen 2x master mix (ready-to-use). Each PCR reaction consisted of 1 µL of each primer, 10 µL exTen 2x ready to use master mix, 6 µL nuclease-free water and 2 µL DNA sample with a final reaction volume of 20 µL. The following amplification conditions were used: initial denaturation at 94°C for three minutes, followed by 30 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, and a final extension at 72°C for five minutes.

The amplified PCR products were subjected to gel electrophoresis [1.0% gel stained with florosafe DNA stain (0.5 µg / ml)] at 70 V for 45 minutes. The gel was placed in an image analyser (Syngene, Cambridge, UK) and the Genesnap software was used to record images and analyse the bands. Bands with a size of 439 bp were considered positive for the *bla*$_{NDM-1}$ gene.

Statistical analysis

Data was compiled and analysed using IBM SPSS Version 26 (IBM Corp, Armonk, NY, USA). Descriptive analysis such as frequency and percentage was

Table I : Primer sequences used for the detection of *bla*$_{NDM-1}$ in *Klebsiella pneumoniae* isolated from patient samples

Targeted gene		Sequence (5′ to 3′)	Length (bases)	Amplicon size (bp)	TM (°C)	Concentration (pmol/µL)
bla$_{NDM-1}$	Forward	TAAAATACCTTGAGCGGGC	19	439	52	15
	Reverse	AAATGGAAACTGGCGACC	18	439	52	15

Source: Mlynarcik et al., (8)

Table II : Socio-demographic data of patients

Demography	Frequency, *n*	Percentage, (%)
Gender		
Male	122	57.3
Female	91	42.7
Age range (years)		
≤ 20	9	4.2
21- 40	28	13.1
41- 60	57	26.8
61- 80	109	51.2
≥ 81	10	4.7
Ethnicity		
Malay	205	96.2
Chinese	5	2.5
Others	3	1.5

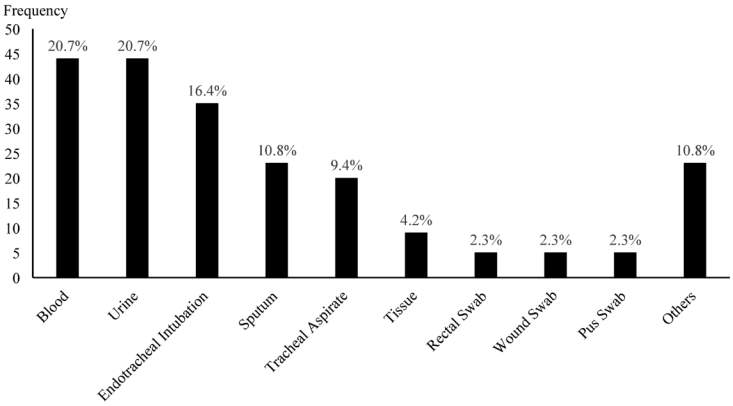

Figure 1 : Distribution of carbapenem resistant *Klebsiella pneumoniae* according to the type of specimens.

performed for all variables. Pearson's Chi-Squared test was used to determine if there were significant associations between socio-demographic variables and the presence of *bla*$_{NDM-1}$ ($p < 0.05$).

Table III : Detection of carbapenem resistance and carbapenemase in bacterial isolates

Test	Frequency (%)	
	Positive	Negative
Carbapenem Resistance		
Imipenem	200 (93.9)	13 (6.1)
Ertapenem	213 (100.0)	0 (0)
Meropenem	189 (88.7)	24 (11.3)
Modified Hodge Test	183 (86.1)	30 (13.9)

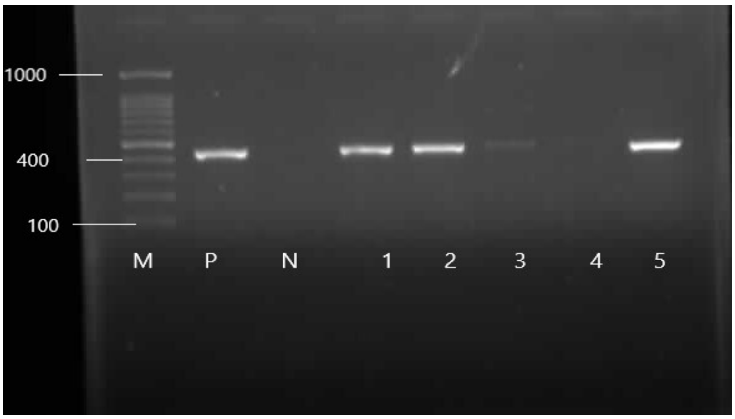

Figure 2 : Agarose gel electrophoresis of *bla*$_{NDM-1}$ gene. M represents 100bp DNA marker ladder, P is positive control, N is negative control and the lines 1-5 represent samples.

RESULTS

Sociodemographic characteristics and sample distribution

The sociodemographic data of patients whose clinical specimens were recruited for this study are shown in Table II. Most of the samples in this study were collected from Malay patients (96.2%, n=205) who were males (57.3%, n=122) ranging from 61 to 80 years old (51.2%, n=109). Figure 1 shows the distribution of CRKP isolated from various clinical samples, where the most common sources were blood (20.7%, n=44), followed by urine (20.7%, n=44), endotracheal intubation (16.4%, n=35) and

Table IV : Association between sociodemographic characteristics and *bla*$_{NDM-1}$ detection

Demography	Frequency (%) of *bla*$_{NDM-1}$ detection		p-value
	Positive	Negative	
Gender			
Male	113 (56.8 %)	9 (64.3)	0.781
Female	86 (43.2 %)	5 (35.7)	
Age range (years)			
≤ 20	9 (4.5)	0 (0)	0.746
21- 40	27 (13.6)	1 (7.1)	
41- 60	55 (27.6)	2 (14.3)	
61- 80	99 (49.7)	10 (71.4)	
≥ 81	9 (4.5)	1 (7.1)	
Ethnicity			
Malay	191 (96.0%)	14 (100)	0.508
Chinese	5 (2.5%)	0 (0)	
Others	3 (1.5%)	0 (0)	

Statistical test – Pearson Chi square

tracheal aspirates (9.4%, n=20). Other clinical specimens comprised 10.8% (n=23), which included bile, pleural fluid, bone tissue etc.

Antibiotic susceptibility and carbapenemase detection
The result of carbapenem resistance revealed susceptibility to ertapenem, imipenem and meropenem, with ertapenem having the highest susceptibility at 100%, imipenem at 94% and meropenem at 89%. All isolates of CRKP that tested positive to carbapenem susceptibility were subjected to confirmatory test using MHT. Out of 213 isolates, a total of 183 (86.1%) were MHT positive and 30 (13.9%) were MHT negative (Table III).

PCR results of *bla*$_{NDM-1}$ gene
Over the period of this study, the *bla*$_{NDM-1}$ gene was detected in 199 (93.4%) of 213 CRKP isolates. Figure 2 shows an example of agarose gel electrophoresis with bands of *bla*$_{NDM-1}$ gene amplicons. Isolates from males, 113 (56.8%) had a higher frequency of *bla*$_{NDM-1}$ than isolates from females, 86 (43.2%). The *bla*$_{NDM-1}$ gene of CRKP isolates were found to be highest in patients aged between 61 to 80 (49.7%, n=99). However, no association was found between any of the socio-demographic variables and the presence of *bla*$_{NDM-1}$ (p>0.05) (Table IV).

DISCUSSION

The high prevalence of *bla*$_{NDM-1}$ gene among CRKP isolates in this study was found to be similar to the rates reported by previous studies (5, 6, 12). One of the studies detected 16 (89%) *bla*$_{NDM-1}$ positive *Klebsiella pneumoniae* isolates out of the 18 samples in their study (6). Whereas, Yeow (12) reported majority of isolated samples in his study were New Delhi metallo-β-lactamase (NDM)-producing carbapenem-resistant Enterobacteriaceae (CRE) (59.3%, 102/172). Data from the national surveillance report on antibiotic resistance, Malaysia (2016) showed that the rate of resistance of *Klebsiella pneumoniae* to carbapenems had increased from 3.2% to 4.0% in 2015 and 3.7% to 4.4% in 2016 (13). Thus, the significant rise of CRKP prevalence rates underscored the need for controlling further spread the bacteria. NDM is an enzyme that is capable of hydrolysing the β-lactam rings in penicillin- and cephalosporin-based antibiotics, rendering them ineffective in treating infections. NDM-1 was first discovered in a strain of *Klebsiella pneumoniae* isolated from a Swedish patient, who was hospitalised in 2008 in New Delhi, India (14).

Most of the CRKP isolates in this study were from blood

and urine samples. This is consistent with previous studies in Malaysia (12) and Turkey (15), which found that the highest number of isolates were collected from blood and urine. One of the reasons why blood and urine samples had more isolates was because *Klebsiella pneumoniae* was a significant Enterobacteriaceae in hospitals that could cause a variety of infections in blood and respiratory and urinary tracts (16).

All the CRKP isolates found in this study were susceptible to ertapenem. This correlated with a previous study in Malaysia that showed CRKP isolates had the highest reduced susceptibility towards ertapenem (n=18; 100%), followed by meropenem (n=17; 94%) and imipenem (n=16; 89%) (5). A study in Egypt showed a similar trend of resistance in CRE towards imipenem, meropenem and ertapenem (97%, 97% and 91%, respectively) (16). A study in China reported that prior use of carbapenems was a risk factor in CRKP infections, although in this study such information was not obtained (18).

As majority of the population in east coast states are Malays living in urban and rural areas, it was expected that this hospital, which is a public institution, would receive a majority of Malay patients, especially those from the middle- and lower-income group. In this study, patients aged between 61 to 80 were found to have the highest prevalence of bla_{NDM-1} bacterial isolates. This result was similar to a United States study that found the median age of hospitalised patients infected with CRKP to be 66 years (19). On the other hand, in the case of CRE, a study in Egypt found that the median age was 19 years (20). The high prevalence of infection among older individuals might be attributed to the patients' weakened immune system, their long-term hospital stay and use of invasive procedures such as catheterisation (21). The frequent movement of older patients across hospitals wards might also play a significant role in the intra-facility spread of CRKP (22). This study attempted to associate the socio-demographic factors (sex, age and ethnicity) and presence of bla_{NDM-1} gene in CKRP isolates at a teaching hospital in a northeast state of Peninsular Malaysia, which found no significant associations. There was a similar study in Nigeria (23), which also reported that gender, age and ethnicity were not risk factors in CRE infections. But they observed that the patients' level of education was significantly associated with CRE infection (p=0.004), which was interesting and needed more study to determine its role. Constant monitoring is crucial as antimicrobial resistance in *Klebsiella pneumoniae* is a serious problem. Hence, it is important to design a prevention and control strategy to address this public health hazard such as health promotion and risk communication, proper and continuous training among healthcare workers on effecteive hand hygiene and universal precaution to prevent nosocomial infection. This can also be accomplished thorugh a signaling system at the hospital by establishing active surveillance to collect epidemiological data, the establishment of a ward for CRKP positive patients, and the formulation of regulations for new antibiotics

CONCLUSION

The prevalence of bla_{NDM-1} gene among CRKP isolates is high and increasing, highlighting the need for control measures. Blood and urine samples have the highest number of isolates, and older patients are more susceptible. All CRKP isolates in this study were susceptible to ertapenem. Socio-demographic factors do not appear to be a significant risk factor for CRE infection. More study is needed to determine the role of patients' education level. Despite the increasing prevalence of bla_{NDM-1}-positive CRKP in Malaysia as obtained in this study, there is a lack of sufficient epidemiological data in the hospital to understand the factors contributing to its high transmission rate in hospitals. Hence, there is an urgent need for further research involving multiple hospitals and detailed patient information, including income level, occupation, and area of residence, to better understand the extent and underlying mechanisms of this resistance.

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REFERENCES

1. Shanthi M, Sekar U, Arunagiri K, Goverdhandas BH. OXA-181 beta lactamase is not a major mediator of carbapenem resistance in Enterobacteriaceae. Journal of Clinical and Diagnostic Research. 2013;7(9):1986–1988. https://doi.org/10.7860/JCDR/2013/5884.3379
2. Bourafa N, Chaalal W, Bakour S, Lalaoui R, Boutefnouchet N, Diene SM, Rolain JM. Molecular characterisation of carbapenem-resistant Gram-negative bacilli clinical isolates in Algeria. Infection and Drug Resistance. 2018;11:735–742. https://doi.org/10.2147/IDR.S150005
3. Netikul T, Kiratisin P. Genetic characterisation of carbapenem-resistant Enterobacteriaceae and the spread of carbapenem-resistant *Klebsiella pneumoniae* ST340 at a university hospital in Thailand. PLoS ONE. 2015;10(9):1–14. https://doi.org/10.1371/journal.pone.0139116
4. Yakubu A, Anua SM, Suraiya S, Mazlan N. A current review on prevalence and molecular charaterisation of carbapenem resistant *Klebsiella pneumoniae* in Malaysia. Malaysian Journal of Medicine and Health Sciences. 2021;17(SUPP8): 233-238

5. Zainol Abidin NZ, Sulong A, Alfizah H, Muttaqillah NA DC. Molecular detection of the New Delhi metallo- b -lactamase-1 gene in Enterobacteriaceae isolates in a tertiary medical centre. Malaysian Journal of Pathology. 2015;37(3):227–32.
6. Zaidah AR, Mohammad NI, Suraiya S, Harun A. High burden of carbapenem-resistant Enterobacteriaceae (CRE) faecal carriage at a teaching hospital: Cost-effectiveness of screening in low-resource setting. Antimicrobial Resistance and Infection Control. 2017; 6(1):42.
7. Wayne P. Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. CLSI Document M100-S24. M100-S24. 2014.
8. Cury AP, Andreazzi D, Maffucci M, Caiaffa-Junior HH, Rossi F. The modified Hodge test is a useful tool for ruling out *Klebsiella pneumoniae* carbapenemase. Clinics. 2012;67(12): 1427–1431. https://doi.org/10.6061/clinics/2012(12)13
9. Ibrahim AH, Omer AFA. Phenotypic detection of carbapenemase-producing *K. pneumoniae* isolated from patients attending hospital settings in Khartoum. African Journal of Medical Sciences. 2018;3(2)ajmsc.info
10. Oliveira CF, De, Paim TG. Da S, Reiter KC, Rieger A, D'azevedo, PA. Evaluation of four different DNA extraction methods in coagulase-negative staphylococci clinical isolates. Revista Do Instituto De Medicina Tropical De Sro Paulo. 2014;56(1):29-33.https://Doi.Org/10.1590/S0036-46652014000100004
11. Mlynarcik P, Roderova M, Kolar M. Primer evaluation for PCR and its application for detection of carbapenemases in Enterobacteriaceae. Jundishapur Journal of Microbiology. 2016; 9(1):e29314. https://doi.org/10.5812/jjm.29314
12. Yeow KK. Epidemiology study of carbapenam-resistant Enterobacteriaceae (CRE): A 30-months experience at a tertiary centre in Northern Malaysia. International Journal of Infectious Diseases, 2020; 101 (Supplement 1): 69, DOI:https://doi.org/10.1016/j.ijid.2020.09.211
13. Rahman A. Carbapenem-resistant Enterobactericeae: clinico-epidemiological perspective. Tropical Biomedicine, 2018;35(2):300–307.
14. Poirel L, Revathi G, Bernabeu S, Nordmann P, Paris Sud M. Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya. Antimicrobial Agents and Chemotherapy. 2011;55(2):934–936. https://doi.org/10.1128/AAC.01247-10
15. Baran I, Aksu N. Phenotypic and genotypic characteristics of carbapenem-resistant Enterobacteriaceae in a tertiary-level reference hospital in Turkey, Annals of Clinical Microbiology and Antimicrobials. 2016;15:20. doi: 10.1186/s12941-016-0136-2
16. Lin D, Chen J, Yang Y, Cheng J, Sun C. (2018). Epidemiological study of carbapenem-resistant *Klebsiella pneumoniae*. Open Medicine. 2018;13:460–466.
17. Mohamed T, Yousef LM, Darweesh EI, Khalil AH, Meghezel EM. Detection and characterisation of carbapenem resistant Enterobacteriacea in Sohag University Hospitals. Egyptian Journal of Medical Microbiology. 2018;27(4):61–69.
18. Guanqun D, Yanling X, Hui K, Weiping X, Hong W. Risk factors for carbapenem-resistant *Klebsiella pneumoniae* infection and associated clinical outcomes. American Journal of Translational Research. 2021; 13(6): 7276–7281.
19. Guh AY, Limbago BM, Kallen AJ. Epidemiology and prevention of carbapenem-resistant Enterobacteriaceae in the United States. Expert Review of Anti-Infective Therapy. 2014;12(5), 565–580. https://doi.org/10.1586/14787210.2014.902306
20. Kotb S, Lyman M, Ismail G, Abd El Fattah M, Girgis SA, Etman A et al. Epidemiology of carbapenem-resistant Enterobacteriaceae in Egyptian intensive care units using National Healthcare-associated Infections Surveillance Data, 2011-2017. Antimicrobial Resistance and Infection Control. 2020;9(1):2.https://doi.org/10.1186/s13756-019-0639-7
21. Agbo EC, Eze EA. Phenotypic detection of metallo-beta-lactamases production in imipenem resistant isolates of *Escherichia coli* and *Klebsiella* species isolated from hospital and environmental. Journal of Biology and Nature. 2015;2(4):135–141.
22. Barnes SL, Harris AD, Golden BL, Wasil EA, Furuno JP. (). Contribution of interfacility patient movement to overall Methicillin-Resistant *Staphylococcus aureus* prevalence levels. Infection Control & Hospital Epidemiology. 2011;32(11):1073-1078 https://doi.org/10.1086/662375
23. Mohammed Y, Zailani SB, Onipede AO, Gadzama GB, Ibrahim BS. Predictors for the acquisition of carbapenem resistance enterobacteriaceae (CRE) from a teaching hospital of Northeastern Nigeria. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research. 2017;05(03):18654-18659