Healthcare associated infections caused by plasmid-encoded bla\textsubscript{KPC} and bla\textsubscript{NDM} strains of \textit{Klebsiella pneumoniae} in Ibadan, Nigeria

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**Introduction:** Carbapenemase producing \textit{Klebsiella pneumoniae} (CPKP) has recently emerged as major cause of healthcare associated infections (HAIs). These strains are now classified by the US Center for Disease Control as superbugs of urgent concern. This study investigated the occurrence of CPKP in HAIs among hospitalized patients in University College Hospital (UCH), Ibadan.

**Methods:** Non-duplicate specimens obtained from 250 patients with clinical HAIs were cultured by standard microbiological methods over a period of 6 months. \textit{K. pneumoniae} was identified using GNB 24E Microbact kit and invitro susceptibility to selected antibiotics was performed by the disk diffusion test. Carbapenemase production was tested by Modified Hodge technique. \textit{K pneumoniae} carbapenemase (bla\textsubscript{KPC}) and New Delhi Metallo-β-lactamase (bla\textsubscript{NDM}) genes were detected by conventional PCR assay. Data were analyzed for the 50 (20%) patients who were culture positive for \textit{K pneumoniae} infections.

**Results:** Isolates were resistant to ampicillin (100%), cefuroxime (96%), amoxicillin (92%), gentamicin (86%), nitrofurantoin (80%), ciprofloxacin (80%), ceftazidime (78%) and ofloxacin (72%) but 76% were sensitive to meropenem. No carbapenemase enzyme was detected but 8 of 12 isolates resistant to meropenem carried bla\textsubscript{KPC} while 2 carried both bla\textsubscript{KPC} and bla\textsubscript{NDM} genes. The study shows that bla\textsubscript{KPC} and bla\textsubscript{NDM} \textit{K pneumoniae} are involved in clinical infections in Ibadan.

**Conclusion:** This study reports the second case of \textit{K pneumoniae} NDM clinical infection in Nigeria. There is need to strengthen the infection control programme of Nigerian hospitals to prevent nationwide spread of these organisms.

**Keywords:** phenotypic, KPC, NDM, \textit{Klebsiella pneumoniae}, infective
1.0 INTRODUCTION

The genus *Klebsiella* consists of encapsulated Gram-negative bacilli that exist as normal bacteria flora of the mouth, skin and intestines of mammals although it is ubiquitously found in surface water, sewage and soil [1]. They are non-motile, lactose fermenting and facultatively anaerobic with tendency to cause destructive changes if they gain access to the human lungs [2]. The *Klebsiella* grow readily on ordinary media commonly used to isolate Enterobacteriaceae such as nutrient agar, trypticase casein soy agar, Bromocresol purple lactose agar, blood agar, as well as more differential plating media such as Drigalski agar, MacConkey agar, eosin-methylene blue agar (EMB), and Bromo-thymol blue agar (BTB). Friedlander Uber in 1882 was the first to discover *Klebsiella* as a human pathogen that caused pneumonia [3]. *Klebsiella pneumoniae* as the most significant member of the genus is commonly found in the gastrointestinal tract and hands of hospital personnel [4]. The pathogenicity of *K. pneumoniae* is due to the possession of a thick capsule layer that surrounds the bacterium [5].

Carbapenems are beta-lactam antibiotics first derived from *Streptomyces cattleya* [6] with broad spectrum antibacterial activity. The unique structure of carbapenems enables them bind with high affinity to beta-lactamase leading to acylation and deactivation of the bacterial enzyme. Since their introduction for clinical use, they have remained one of the antibiotics of last resort for treatment of many bacterial infections [7], and particularly considered to be agents of choice for treatment of infections caused by the extended spectrum beta-lactamase (ESBLs) producing enterobacteriaceae.

Some of the drugs belonging to the carbapenem class that have been approved for use include imipenem, meropenem, panipenem/betamipron, ertapenem, doripenem and biapenem [8]. However, resistance to carbapenems among family Enterobacteriaceae especially *Klebsiella pneumoniae* and *Escherichia coli* has emerged worldwide (9). Although several mechanisms of resistance to carbapenems have been reported (10), the carbapenemases, which are versatile beta–lactamas of classes A, B and C that are capable of inactivating carbapenems, have been the most prominent enzymes involved, and *K. pneumoniae* carbapenemase (KPC) which belongs to class A is the most prevalent (10). As there are currently no new antibiotics in development to combat bacteria resistant to carbapenems, the worldwide spread of these resistant strains is considered a potential nightmare scenario [11].

The aim of this study therefore is to determine the occurrence of carbapenem resistance in *K. pneumoniae* clinical isolates from hospitalized patients. The objectives are to; (i) isolate *K. pneumoniae* from clinical samples collected from hospitalized patients in University College Hospital, Ibadan, Nigeria; (ii) determine the in-vitro susceptibility of isolated *K. pneumoniae* to selected antibiotics; (iii) detect carbapenemase activity, and (iv) perform polymerase chain reaction assay to detect carbapenemase genes in the isolated *K. pneumoniae*.

2.0 METHODOLOGY

2.1 Study location and design

The study was carried out at the University College Hospital, Ibadan and Medical Microbiology and Parasitology Laboratory, College of Health Sciences, Ladoke Akintola University of Technology, Osogbo, Southwestern Nigeria.

This research is a descriptive cross sectional study carried out over a 6 month period, August 2015 to January 2016. The subject participants were hospitalized patients with healthcare associated infections (HAIs) from University College Hospital, Ibadan, Nigeria.

2.2 Ethical issues

Informed consent was obtained from each patient-participant, and ethical approval of the hospital management was obtained prior to conduct of the study.

2.3 Sampling, specimen and data collection

Non-repetitive routine clinical samples (sputum, blood, urine, wound swabs and tracheal aspirates) from a total of 250 eligible hospitalized patients with suspected clinical infections in different wards of the hospital were collected over the study period. Relevant clinical data for analysis were retrieved for only 50 patients confirmed to have *K. pneumoniae* infections, and entered into a recording form.

2.4 Culture and presumptive identification

All fresh clinical specimens (sputum, urine, wound swabs and tracheal aspirates) were directly cultured on Blood and MacConkey agar plates at the Medical Microbiology Laboratory of the University College Hospital, Ibadan, Nigeria to isolate *Klebsiella* species. Blood specimens were collected into aerobic Blood culture bottles and first incubated overnight before sub-culture on Blood and MacConkey agar plates. The culture plates were incubated at 370C aerobically for 24 hours and *Klebsiella* isolates were presumptively identified as mucoid, lactose fermenting colonies [12]. Subculture of each isolate was done on Nutrient agar slant and then transported to the Medical Microbiology Laboratory of the College of Health Sciences, LAUTECH, Osogbo, for further analysis.
2.5 Biochemical identification of *K. pneumoniae* with Microbact kit (GNB 24E)

The GNB 24E Microbact Kit (Oxoid, England) was used to biochemically characterize presumptively identified *Klebsiella* isolates to species level. This was achieved by making suspensions of the different isolates in test tubes under aseptic condition and adjusting them to 0.5 McFarland standards in normal saline water. The wells (containing lyophilized form of the biochemical reagents for the different tests) of the Microbact kit plates were filled with the bacterial suspensions and incubated at 37°C for 24 hours. To well 8 (indole production), two drops of indole (Kovacs reagent) was added and the result evaluated within 2 minutes of the addition of the reagent. To well 10 (Vogues-Proskauer reaction), 1 drop of VPI and VPII reagents were added and result evaluated within 15 to 30 minutes after the addition of reagents. To well 12 (tryptophan deaminase), 1 drop of TDA reagent was added and evaluated immediately after the addition of the reagent. Each reaction was interpreted as positive or negative after comparing with the colour chart. The identity of each *K. pneumoniae* isolate was subsequently determined after inputting the test results into the octal code of the Microbact computer software (Oxoid, England). *Escherichia coli* NCTC 10418 was used as positive control and *Proteus mirabilis* NCTC 10975 as negative control strain.

2.6 Modified Hodge Test (MHT) for carbapenemase

The modified Hodge test was used to screen for carbapenemase in each *K. pneumoniae* isolate [13]. Briefly, 0.5 McFarland standard dilution of the E. coli ATCC 25922 (indicator organism) was prepared in 5 ml of Mueller-Hinton Broth (MHB). One in 10 dilution of this was then prepared and streaked onto MH agar plate. Ten µg meropenem disk was placed in the centre of the test area and in a straight line. *K. pneumoniae* test isolate was streaked from the edge of the disk to the edge of the plate and the control *K. pneumoniae* strains in another direction. The MH plates were then incubated at 37°C aerobically for 24 hours after which they were examined for the presence or absence of a clover leaf-type indentation at the intersection of *K. pneumoniae* test isolate and the indicator organism within the zone of inhibition of the meropenem disk. The presence of clover-leaf indentation indicates a positive MHT (carbapenemase produced) and absence of clover-leaf indentation indicates a negative MHT (no carbapenemase produced). Positive control strain used was *K. pneumoniae* ATCC® BAA-1705™ while negative control strain was *Klebsiella pneumoniae* ATCC® BAA-1706™.

2.7 Disk diffusion susceptibility testing of isolates

The invitro susceptibility of each *K. pneumoniae* isolate to selected antibiotics was performed using the disk diffusion test of Bauer et al., [14]. The diameter of zone of inhibition of each isolate to the disk was compared with interpretative standards of the CLSI [13] to determine sensitivity or resistance. Control strain used was *Escherichia coli* ATCC 25922.

2.8 Molecular detection of KPC and NDM genes by PCR assay

The conventional simplex polymerase chain reaction (PCR) assay was used to detect KPC and NDM genes in phenotypically confirmed *K. pneumoniae* isolates. DNA extraction was done with slight modification of the boiling method [15]. Briefly, a sub-culture of *K. pneumoniae* isolates was first made on Nutrient agar plate which was incubated at 37°C for 24 hours. Four to five pure colonies of each isolate were then then streaked from the plate into an appropriately labelled Eppendorf tube filled with 500 µL of TBE buffer. The tubes were covered and sealed with paraffin tape to prevent accidental opening. The bacterial suspensions were then boiled at 100°C for 7 minutes in a water bath, cooled on ice, and then centrifuged at 15,000 x g for 30 seconds. The supernatant containing the DNA was stored at 4°C before use. Aliquots of 5 µL of template DNA were used for PCR.

Amplification of bla*KPC* genes (KPC 1 to KPC 5) was done with multi-KPC forward primer, *caaacaggtgctttcctgct* and multi-KPC reverse primer, *agcagccataatcattgt*- cattg which amplified a 538 bp fragment [16]. Amplification of the entire sequence of bla*NDM* genes was done with pre-NDM forward primer, *cacctctatgttggaatg* and pre-NDM reverse primer, *ctctgtcaagacggcata*- gac which gave a 561 bp amplicon [17, 18]. The reaction was set up in a PCR tube after addition of the forward and reverse primers, and extracted DNA. A 25 µl of master mix contained 4 µl of 10x buffer, 0.5 µl MgCl2, 3 µl dNTPs and 0.2 µl *Taq* polymerase. The PCR vial was placed in the Thermocycler (Prime thermal cycler, UK) and programmed to initial denaturation at 95°C for 10 min, then 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds and extension at 72°C for 30 seconds. A final extension procedure was carried out at 72°C for 10 min.

The amplicons were resolved on 1.5 % agarose gel into which 0.5 µl of ethidium bromide (EtBr) had been added. Agarose plates were placed inside the electrophoretic tank (Weal Tek Corp, Taiwan) which contains 1 x TBE solution. 5 µl of each amplicon was mixed with 5 µl of orange G (loading buffer) and was loaded into the appropriate wells and allowed to run for 30 mins at 100 volts.
For each run, a 100 base-pair molecule weight DNA standard (New England Biolabs “NEB”) was used to verify the proper size for each PCR product. The DNA bands were then captured and visualized with a shortwave ultraviolet transilluminator and photographed using gene gel bio-imaging system (UVP imaging system, Upland, CA, USA).

2.9 Data analysis

All data (demographic and clinical) for the 50 patients were entered into Excel sheet for statistical analysis using SPSS version 20.0. Frequency tables were generated and relationship between variables tested with Chi square with significant value set at P< 0.05.

3.0 RESULTS

*Klebsiella pneumoniae* isolates were recovered from different clinical samples of 50 (20%) patients out of the 250 eligible hospitalized patients with different invasive healthcare associated infections at the University College Hospital, Ibadan, Nigeria, during the period of study. Table 1 shows the gender and age group distribution of the patients, with age group 10–19 years constituting the largest percentage (26%), followed by age group ≥ 70 years (22%), 20–29 years (16%), 50–59 years and 60–69 years (10%), and 20–29 and 30–39 years (8%).

Table 1: Age group and gender distribution of patients from whom *Klebsiella pneumoniae* was isolated

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Male</th>
<th>Female</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10–19</td>
<td>8</td>
<td>5</td>
<td>13 (26)</td>
</tr>
<tr>
<td>20–29</td>
<td>5</td>
<td>3</td>
<td>8 (16)</td>
</tr>
<tr>
<td>30–39</td>
<td>1</td>
<td>3</td>
<td>4 (8)</td>
</tr>
<tr>
<td>40–49</td>
<td>3</td>
<td>1</td>
<td>4 (8)</td>
</tr>
<tr>
<td>50–59</td>
<td>2</td>
<td>3</td>
<td>5 (10)</td>
</tr>
<tr>
<td>60–69</td>
<td>2</td>
<td>3</td>
<td>5 (10)</td>
</tr>
<tr>
<td>≥70</td>
<td>7</td>
<td>4</td>
<td>11 (22)</td>
</tr>
<tr>
<td>Total</td>
<td>29 (58%)</td>
<td>21 (42%)</td>
<td>50 (100)</td>
</tr>
</tbody>
</table>

Table 2 shows clinical diseases and specimen types collected from the patients. Pneumonia was the most frequent infection (46.0%), followed by urinary tract infection (24.0%), wound and surgical site infection (20.0%), bacteremia (6.0%), and chronic obstructive pulmonary disease (4.0%).

Table 2: Clinical disease and specimen of patients infected with *Klebsiella pneumoniae*

<table>
<thead>
<tr>
<th>Clinical disease</th>
<th>Specimen</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia</td>
<td>Sputum</td>
<td>19 (38.0)</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>Trachea aspirate</td>
<td>4 (8.0)</td>
</tr>
<tr>
<td>Wound infection</td>
<td>Sputum</td>
<td>2 (4.0)</td>
</tr>
<tr>
<td>Surgical site infection</td>
<td>Wound swab</td>
<td>6 (12.0)</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>Urine</td>
<td>12 (24.0)</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>Blood</td>
<td>3 (6.0)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>50 (100)</td>
</tr>
</tbody>
</table>

Table 3 displays the in vitro antibiotic susceptibility of *K. pneumoniae* isolates. Of the 50 isolates, 38 (76%) were sensitive to meropenem while 12 (24%) were resistant. The isolates were largely resistant to commonly used antibiotics in this environment; ampicillin (100%), cefuroxime (96%), amoxicillin (92%), gentamicin (86%), nitrofurantoin (80%), ciprofloxacin (80%), ceftazidime (78%) and ofloxacin (72%).

Table 4 shows the susceptibility pattern of meropenem resistant *K. pneumoniae* isolates. Of the 12 isolates, 8 (66.7%) were positive for KPC gene and 2 out of these were also positive for NDM gene. The 8 isolates were 100% resistant to ampicillin, amoxicillin, cefuroxime and ciprofloxacin, 87.5% to ceftazidime, 87% to ofloxacin, and 75% to each of gentamicin and nitrofurantoin. The 2 isolates that carried both KPC and NDM genes were resistant to all antibiotics tested. None of the 12 isolates produced carbapenemase by the MHT test.

Fig 1 shows the gel electrophoresis of the amplified KPC gene (536 bp) for 8 of the 12 meropenem resistant *K. pneumoniae* isolates (isolates 1, 2, 3, 4, 5, 7, 8 and 10). Fig 2 shows a representative isolate that amplified for NDM gene (561 bp).

4.0 Discussion

In this study, *K. pneumoniae* isolates demonstrated multiple resistance to the antibiotics tested, which agrees with worldwide reports of increase resistance to antibiotics among *Klebsiella* spp [19]. Resistance to ampicillin was 100%, and this is similar to the reports of other studies that recorded such high resistance rate [20-24].
The cephalosporins, particularly the second and third generations, have been employed as the drug of choice for treating infections caused by the family enterobacteriaceae including *Klebsiella* infections [25]. In our study however, 78% of the *K. pneumoniae* isolates were resistant to ceftazidime, a third generation cephalosporin. Previous studies by Ullah et al. [21], Eze [26], Ejikeugwu et al., [27] and Sanjay et al., [28] have also reported high resistance rate among *K. pneumoniae* to ceftazidime. The resistance rate for cefuroxime, another third generation cephalosporin, was 96% which is much higher than reports from India [24]. Although, the aminoglycosides such as gentamicin have been known to have good activity against clinically important gram negative bacilli [29], only 14% of the isolates were susceptible to gentamicin in this study. This agrees with 16.7% and 17.4% susceptibility rates reported in studies carried out in India [30] and Pakistan [22] respectively.

**Table 3: Susceptibility pattern of *Klebsiella pneumoniae* isolates**

<table>
<thead>
<tr>
<th><em>Klebsiella pneumoniae</em> (n=50)</th>
<th>Antibiotics (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amp (%)</td>
</tr>
<tr>
<td>S</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>50 (100)</td>
</tr>
</tbody>
</table>

Amp = Ampicillin, Amox = Amoxicillin, Cefu = Cefuroxime, Ceft = Ceftazidime, Gent = Gentamicin,
Nitr = Nitrofurantoin, Ofx = Ofloxacin, Cipr = Ciprofloxacin, Mero = Meropenem

**Table 4: Susceptibility of *Klebsiella pneumoniae* isolates carrying KPC and NDM genes**

<table>
<thead>
<tr>
<th>Isolates (n=8)</th>
<th>Antibiotics (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amp</td>
</tr>
<tr>
<td>S</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>8 (100)</td>
</tr>
</tbody>
</table>

* 2 of the 8 isolates carry both KPC and NDM genes and were resistant to all antibiotics tested. Amp = Ampicillin, Amox = Amoxicillin, Cefu = Cefuroxime, Ceft = Ceftazidime, Gent = Gentamicin, Nitr = Nitrofurantoin, Ofx = Ofloxacin, Cipr = Ciprofloxacin, Mero = Meropenem

In our study however, 78% of the *K. pneumoniae* isolates were resistant to ceftazidime, a third generation cephalosporin. Previous studies by Ullah et al. [21], Eze [26], Ejikeugwu et al., [27] and Sanjay et al., [28] have also reported high resistance rate among *K. pneumoniae* to ceftazidime. The resistance rate for cefuroxime, another third generation cephalosporin, was 96% which is much higher than reports from India [24]. Although, the aminoglycosides such as gentamicin have been known to have good activity against clinically important gram negative bacilli [29], only 14% of the isolates were susceptible to gentamicin in this study. This agrees with 16.7% and 17.4% susceptibility rates reported in studies carried out in India [30] and Pakistan [22] respectively.

![Fig 1: Gel electrophoresis of PCR amplified products of KPC gene](image-url)
For the fluoroquinolones, resistance rates of *K. pneumoniae* to ciprofloxacin and ofloxacin were 80% and 72% respectively in this study. The resistance rate to ciprofloxacin is higher than 23% reported by Asati [24] and Manikandan et al., [23] and 59% by Ejikeugwu et al., [27]. Asati [24] recorded a much higher resistance rate to ofloxacin of 88.8% than the present study but Manikandan et al., [23] reported a far less rate of 20.8%. Resistance rates appear to vary from location to location depending on level of use of these antibiotics. In Nigeria, antibiotic prescription is not well regulated by Government agencies and are therefore available over-the-counter to the populace at will. This give rooms for antibiotic “self medication” and the attendant misuse leading to emergence of resistant organisms.

There have been worldwide reports of resistance to carbapenems for over a decade in countries such as Israel [31], China [32], South America [33] and France [34]. In our current study, the *in vitro* susceptibility of *K. pneumoniae* isolates to meropenem (a carbapenem) was 76%, with 24% resistance rate. Although this may indicate that meropenem still has good *in vitro* activity against *K. pneumoniae*, when compared with the report of Ejikeugwu et al., [27] who reported 87.2% susceptibility (12.8% resistance) in 2012, our study suggests there is a gradual increase in resistance rates to meropenem in our environment.

All the 12 *K. pneumoniae* isolates in our study that showed phenotypic resistance to meropenem were negative for carbapenemase production by the Modified Hodge test but 8 of them were positive for blaKPC and blaNDM genes. This finding agrees with the report of Okoche et al., [35] for gram negative bacteria isolates that did not produce detectable carbapenemase by MHT but carried blaKPC and blaNDM-1 genes. This is also corroborated by another study in which double disk synergy test (DDST) detected metallo-beta-lactamase (MBL) in 33 of 34 multidrug resistant gram negative bacteria that carried blaNDM-1 gene while MHT detected carbapenemases in only 26 of the 34 isolates [36], with Vitek 2 Compact 60 automated system being the only test that detected carbapenemase in all the isolates in the study. The reason for the poor performance of MHT for detection of carbapenemase in our study is not apparent but MHT has been reported to exhibit low sensitivity (false negative) of 11% in detecting carbapenemase particularly when blaNDM-1 strains are involved (37, 38).

The 8 isolates carrying blaKPC or blaNDM genes in our study were all resistant to ampicillin, amoxycillin, cefuroxime and ciprofloxacin while the 2 that carried both genes were resistant to all the antibiotics tested in the study. Plasmids containing blaNDM-1 and blaKPC genes are known to also carry a number of other genes conferring resistance to aminoglycosides, macrolides and sulphanmethoxazole. Bacteria that harbour these plasmids are therefore usually multidrug resistant and because of their carriage of other non-plasmid-mediated resistance, may be resistant in some cases to all antibiotics [39]. The complete resistance of the two isolates in this study to all tested antibiotics may therefore be a result of their carriage of both blaNDM and blaKPC genes. Although carbapenems (e.g. imipenem and meropenem) have been adjudged to be the best treatment options for multidrug resistant bacterial infections, the resistance to meropenem and to other antibiotics in our study is a cause for concern especially in this era of decreasing pipeline for antibiotic discovery.

**Conclusion**

This study has reported high rate of resistance of *K. pneumoniae* isolates in our environment to some frontline antibiotics among patients with healthcare associated infections. The isolation of *K. pneumoniae* isolates carrying NDM gene, which is the second report from clinical infection in Nigeria after that of Uwaeszuko et al., [40], is a serious concern in this environment as these strains are known to carry resistance genes to multiple antibiotics. Owing to the fact that carbapenems are the last resort in the treatment of ESBL infections and other multidrug resistant gram negative infections, there is an urgent need for constant monitoring of susceptibility of pathogens in different populations to commonly used
antimicrobial agents through detection and surveillance in both the community and hospital settings.

Stringent measures including prompt and accurate detection methods for ESBLs and MBLs, adequate infection control measures and a review of antibiotic guidelines should be introduced in Nigerian hospitals so as to assess the burden of antibiotic resistance and contain their possible emergence and spread. This study further buttresses the need for the establishment of an “antimicrobial resistance detection and monitoring reference laboratory” in Nigeria so that antibiotic resistance cases can be properly detected, reported and contained.

**Conflict of interest**
The authors declare no conflict of interest

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**Contribution of authors**
The authors did not receive funding for the study

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