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ORIGINAL ARTICLE

Horizontal Gene Transfer of *bla*$_{NDM-5}$ Among Three Different Enterobacteriaceae Species Isolated from a Single Patient

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SUMMARY

Background: **In this study, *Escherichia coli*, *Klebsiella oxytoca*, and *Citrobacter amalonaticus* carrying *bla*$_{NDM-5}$ were isolated from a single patient.**
Methods: **The antibiotic susceptibility of the isolates was evaluated by using E-test and agar dilution methods, and *bla*$_{NDM-5}$ was identified in genomic and plasmid DNA by using polymerase chain reaction and sequencing. Whole genome sequencing and de novo assembly were used for species characterization, resistance gene identification, and plasmid analysis.**
Results: **All three species had identical plasmids, which were similar to pEC463-NDM5, a plasmid harboring *bla*$_{NDM-5}$. Transconjugation experiments confirmed the horizontal gene transfer of *bla*$_{NDM-5}$, highlighting the need for a close monitoring of Enterobacteriaceae species harboring this gene.**
Conclusions: **This study conclusively demonstrates the propensity for horizontal gene transfer of *bla*$_{NDM-5}$ among Enterobacteriaceae species, underlining the importance of vigilant monitoring to combat antibiotic resistance.**
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INTRODUCTION

For decades, the spread of *carbapenem-resistant Enterobacteriaceae* (CRE) species has been a serious problem in healthcare [1,2]. CRE species emerge primarily because of the transmission of genes encoding carbapenemase [3,4]. In particular, the increase in the prevalence of CRE species is related to a plasmid-mediated horizontal transmission of carbapenemase-encoding genes [5,6]. New Delhi metallo-beta-lactamase (NDM), which was first reported in 2009, is a carbapenemase that can hydrolyze all beta-lactams, except monobactam [7]. Since 2009, 19 variants of NDM genes and enzymes have been identified [8-11]. NDM-5 was first identified in *Escherichia coli* in a patient who

had been hospitalized in India [12]. It has higher resistance to carbapenems and broad-spectrum cephalosporins than NDM-1 [12]. Some studies have reported that *bla*$_{NDM-5}$ encoding NDM-5 enzyme can be transferred by a plasmid [13,14]. Recently, we isolated *E. coli*, *Klebsiella oxytoca*, and *Citrobacter amalonaticus* carrying *bla*$_{NDM-5}$ from a single patient. We aimed to characterize these three CRE species and to investigate the horizontal gene transfer of *bla*$_{NDM-5}$ among bacterial species.

MATERIALS AND METHODS

Carbapenem-resistant *E. coli* was isolated from a routine screening rectal swab of a 73-year-old female patient with a femur fracture and ankylosing spondylitis. Carbapenem resistance was determined by using the VITEK 2 system (BioMerieux, France) and confirmed by using imipenem and ertapenem disk diffusion tests. The same bacteria were detected in three consecutive examinations. Simultaneously, carbapenem-resistant *K. oxytoca* and *C. amalonaticus* were isolated from rectal swabs, together with the carbapenem-resistant *E. coli*. Antimicrobial susceptibility to carbapenems was determined by using the agar dilution method, according to the guidelines of the Clinical and Laboratory Standards Institute [15]. The presence of carbapenemase-encoding genes (*bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{VIM}$, and *bla*$_{OXA-48}$) was determined and identified via polymerase chain reaction (PCR) and sequencing, with the primers listed in Table 1.

Subsequently, whole genome sequencing (WGS) and data analysis were conducted. Briefly, genomic DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), and DNA quality was confirmed by using gel electrophoresis. Libraries were prepared by using the TruSeq Nano DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and sequenced on a HiSeq 2,500 Sequencer (Illumina). The quality of the raw data was assessed by using FastQC, followed by the de novo assembly with SPAdes (http://cab.spbu.ru/software/spades/) and a taxonomic classification by using the Kraken Taxonomic Classifier (https://ccb.jhu.edu/software/kraken/). Resistance genes and plasmid types were annotated by using ResFinder 4.1 [16] and PlasmidFinder 2.0 [17], respectively, at the Center for Genomic Epidemiology (http://www.genomicepidemiology. org/).

Plasmid DNA was subjected to paired-end WGS with an insert size of 350 bp by using the HiSeq X Sequencer (Illumina, San Diego, CA, USA). The WGS data of plasmid DNA were mapped to a reference sequence by using gsMapper 2.8 (Roche, Basel, Switzerland), and a line map was generated by using the EasyFig 2.2.2 (http://mjsull.github.io/Easyfig/) program (Figure 1). The sequence of pEC463-NDM5 (accession number MG545911), which is known to carry *bla*$_{NDM-5}$ in *E. coli*, was used as a reference for mapping [14].

In the transconjugation experiment, the donor isolates (*E. coli*, *K. oxytoca*, and *C. amalonaticus*) were mixed with the recipient, sodium azide-resistant *E. coli* J53, and cultured on MacConkey agar containing sodium azide and imipenem.

RESULTS

The results of the antimicrobial susceptibility testing indicated that all three species were resistant to ertapenem, imipenem, and meropenem. The PCR results revealed a *bla*$_{NDM}$-type carbapenemase gene in all six DNA samples, and *bla*$_{NDM-5}$ was identified through sequencing.

The raw data obtained by using WGS contained a mean of 6,500,000 reads per isolate. The Phred quality score indicated that all three species had an average of 40 or more over 99%. The Kraken Taxonomic Classifier demonstrated that all three species had a consistent taxonomic classification, as confirmed by using VITEK 2. Furthermore, *bla*$_{NDM-5}$ was detected in all three species by using ResFinder and an IncX3-type plasmid was identified in the species by using PlasmidFinder.

The WGS data of plasmid DNA were mapped to a reference sequence and a line map was generated (Figure 1). The plasmids detected in all three species had the same sequence, except that protein 980 in one plasmid was valine instead of alanine. In addition, the plasmid sequences of the three species had a high similarity to the reference sequence; however, a structural variation was found at the same site (Protein ID: AWI97807.1).

In the transconjugation experiment, bacterial growth was observed on all three plates. Matrix-assisted laser desorption/ionization-time of flight identification confirmed that all isolates were *E. coli*, and PCR and sequencing confirmed the presence of *bla*$_{NDM-5}$ in all three isolates.

DISCUSSION

In this study, we isolated three CRE species, namely *E. coli*, *K. oxytoca*, and *C. amalonaticus*, from one patient, containing the same carbapenemase-encoding gene and nearly genetically identical plasmids. By using NGS, traditional PCR, electrophoresis, and sequencing, the three isolates were confirmed to contain *bla*$_{NDM-5}$ and an IncX3-type plasmid. The plasmid analysis revealed that the IncX3-type plasmid is genetically similar to the pEC463-NDM5 plasmid [14]. Furthermore, by using transconjugation experiments, we demonstrated that *bla*$_{NDM-5}$ was transferred between the species. Resistance genes are transferred horizontally via plasmids [6]. Some studies have shown the presence of *bla*$_{NDM-5}$ in the IncX3 plasmid in Enterobacteriaceae species [13, 14]. The IncX3 plasmids are broad-host-range plasmids that contain iterons and various antibiotic-resistance genes such as *bla*$_{KPC-2}$, *bla*$_{SHV-1}$, and *bla*$_{NDM-1}$ [18,19].

Table 1. Primers used to detect resistance genes.

Primer	Sequence (5′ - 3′)	Amplicon size (bp)	Reference
bla$_{KPC}$_F	CGTCTAGTTCTGCTGTCTTG	798	[20]
bla$_{KPC}$_R	CTTGTCATCCTTGTTAGGCG		
bla$_{NDM}$_F	GGTTTGGCGATCTGGTTTTC	621	
bla$_{NDM}$_R	CGGAATGGCTCATCACGATC		
bla$_{IMP}$_F	GGAATAGAGTGGCTTAAYTC	232	
bla$_{IMP}$_R	GGTTTAAYAAAACAACCACC		
bla$_{VIM}$_F	GATGGTGTTTGGTCGCATA	390	
bla$_{VIM}$_R	CGAATGCGCAGCACCAG		
bla$_{OXA-48}$_F	GCGTGGTTAAGGATGAACAC	438	
bla$_{OXA-48}$_R	CATCAAGTTCAACCCAACCG		

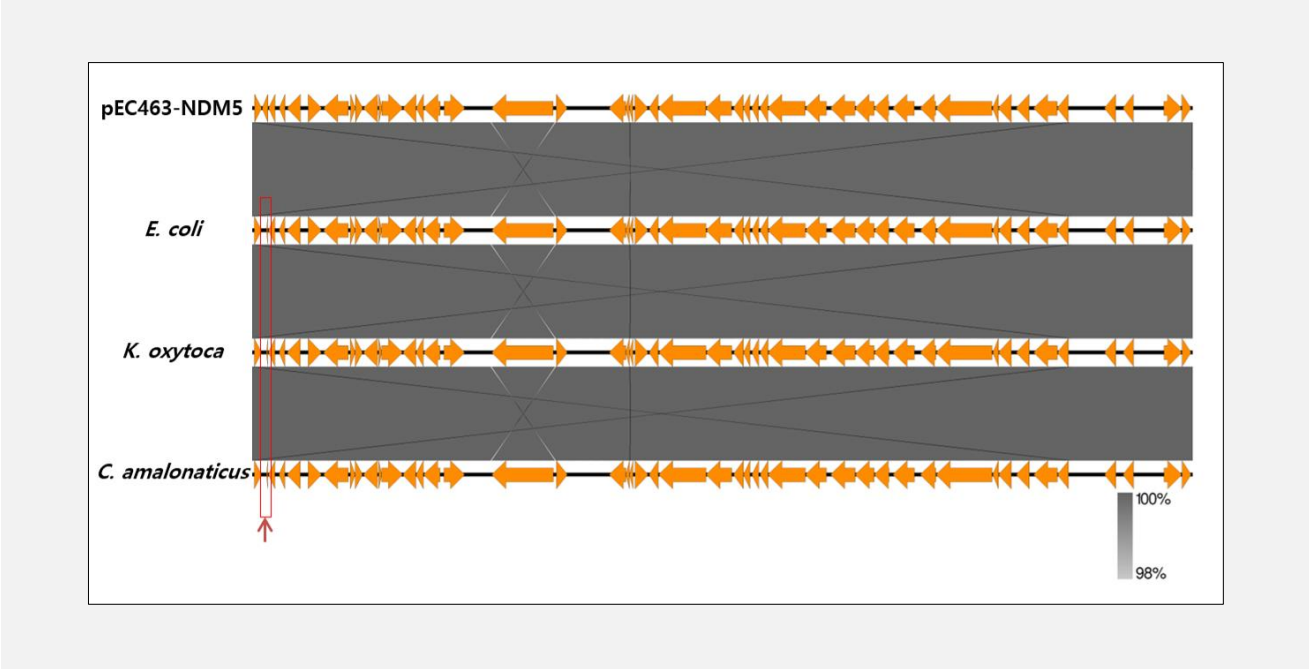

Figure 1. Genome linear maps of pEC463-NDM5 and plasmids from three isolates.

A structural variation different from the reference was found (box and arrow).

To the best of our knowledge, this is the first report of *bla*$_{NDM-5}$ transfer via an IncX3 plasmid in *K. oxytoca* and *C. amalonaticus*. Although we did not conduct experiments beyond PCR and sequencing, we can infer that *bla*$_{NDM-5}$ detected in the transconjugants was transferred from the donor. Despite the findings, this study was limited by the small number of samples. Nevertheless, we observed *in vitro* horizontal gene transfer via the IncX3 plasmid through various experiments. In conclusion, we demonstrated interspecies *bla*$_{NDM-5}$ transfer via an IncX3-type plasmid from a single patient by using NGS. Our findings highlight the need for close surveillance of the transfer of *bla*$_{NDM-5}$ between Enterobacteriaceae species.

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Declaration of Interest:
The authors declare that there is no conflict of interest.

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