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High diversity of bla_{NDM-1}-encoding plasmids in *Klebsiella pneumoniae* isolated from neonates in a Vietnamese hospital

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A R T I C L E   I N F O

A B S T R A C T

Objectives: The carbapenemase-encoding gene bla_{NDM-1} has been reported in Vietnam during the last 10 years, and bla_{NDM}-producing Enterobacteriaceae are now silently and rapidly spreading. A key factor behind dissemination of bla_{NDM-1} is plasmids, mobile genetic elements that commonly carry antibiotic resistance genes and spread via conjugation. The diversity of bla_{NDM-1}-encoding plasmids from neonates at a large Vietnamese hospital was characterized in this study.

Methods: 18 fecal *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae* isolates collected from 16 neonates at a large pediatric hospital in Vietnam were studied using optical DNA mapping (ODM) and next-generation sequencing (NGS). Plasmids carrying the bla_{NDM-1} gene were identified by combining ODM with Cas9 restriction. The plasmids in the isolates were compared to investigate whether the same plasmid was present in different patients.

Results: Although the same plasmid was found in some isolates, ODM confirmed that there were at least 10 different plasmids encoding bla_{NDM-1} among the 18 isolates, thus indicating wide plasmid diversity. The ODM results concur with the NGS data. Interestingly, some isolates had two distinct plasmids encoding bla_{NDM-1} that could be readily identified with ODM. The coexistence of different plasmids carrying the same bla_{NDM-1} gene in a single isolate has rarely been reported, probably because of limitations in plasmid characterization techniques.

Conclusions: The plasmids encoding the bla_{NDM-1} gene in this study cohort were diverse and may represent a similar picture in Vietnamese society. The study highlights important aspects of the usefulness of ODM for plasmid analysis.

1. Introduction

Carbapenem-resistant Enterobacteriaceae (CRE), including *Klebsiella pneumoniae*, play a significant role in hospital-acquired infections (HAIs) and lead to increased morbidity, mortality, and health-care costs [1–3]. An alarming epidemic spread of CRE in hospitals has been reported in many parts of the world, including Southeast Asia. A point prevalence survey of around 5000 patients in 21 Vietnamese ICUs in 2012-2013 showed that 30% had HAIs that were frequently caused by Gram-negative pathogens with high carbapenem resistance rates [3, 4]. In one large Vietnamese neonatal ICU, CRE colonization increased from 32% at admission to 87% at discharge and there was a significant correlation between CRE col-
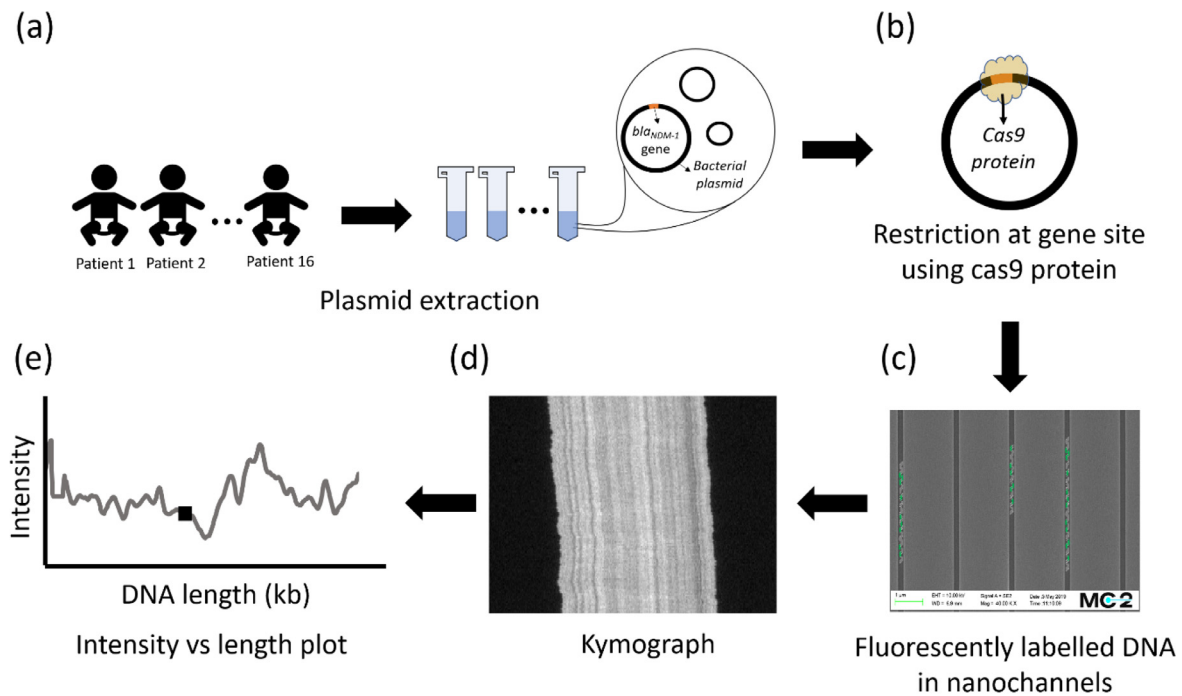
* Corresponding author.

Figure 1. Schematic representation of the study. (a) Bacterial plasmids were isolated from *K. pneumoniae* and *K. quasipneumoniae* isolates from fecal samples from neonates in a large Vietnamese hospital. (b) Plasmids were restricted at the bla_{NDM-1} site using Cas9. (c) Plasmids were labelled in one step using YOYO-1 and netropsin, followed by fluorescence imaging of linearized plasmids in nanochannel devices. (d) Kymographs were generated from the movies collected. (e) Barcodes were obtained by averaging the kymographs. The black square represents the location of bla_{NDM-1}.
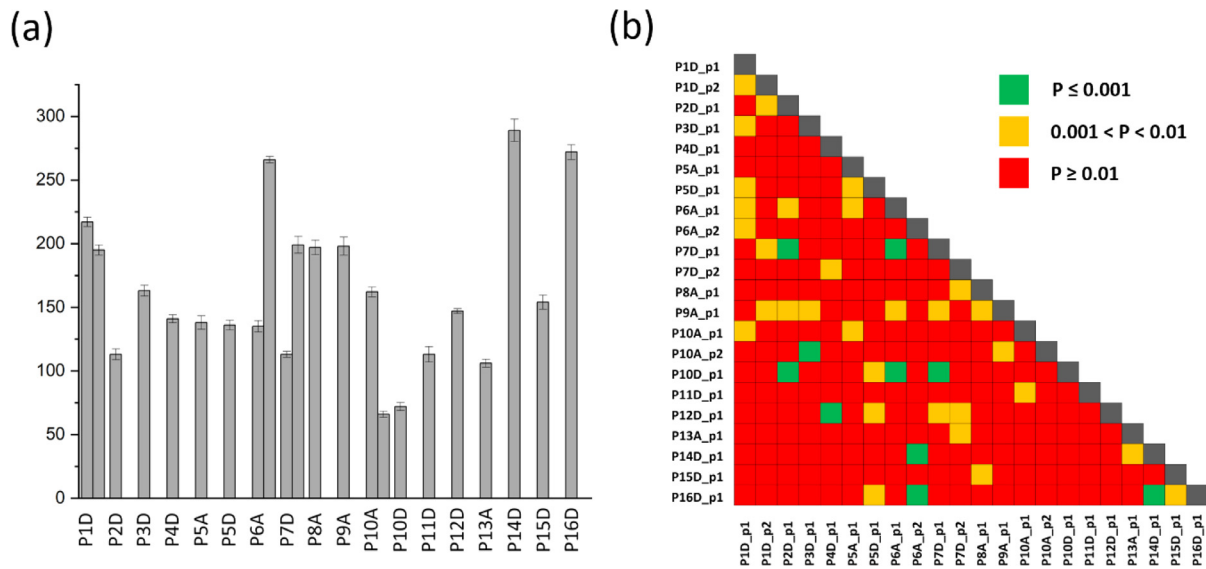

Figure 2. (a) Histogram showing the length of all plasmids (in kilo-base pairs) carrying bla_{NDM-1} in each isolate studied. (b) Similarity matrix of barcodes for plasmids carrying bla_{NDM-1}. A pairwise comparison of the barcodes was performed. Pairs with $P \leq 0.001$ are labelled green and are considered a "good match". Pairs with $0.001 < P < 0.01$ are labelled yellow and are considered a "partial match". Finally, pairs with $P \geq 0.01$ are labelled red and are considered "no match".

onization and HAI, as well as between HAI and mortality [5]. In another study performed in the USA, HAIs caused by carbapenem-resistant *K. pneumoniae* (CRKP) had a crude mortality among children of ~35% [6]. In CRE point prevalence screenings performed at 12 Vietnamese hospitals in 2017-2018, half the 2233 patients were colonized with CRE [5]. Recent findings indicate that screening during admission and cohort care decrease CRE in Vietnamese pediatric ICUs [7].

The bla_{NDM-1} gene encodes the New Delhi metallo-β-lactamase-1 (NDM-1) enzyme that confers resistance to both cephalosporins and carbapenems [8]. During the last decade, bla_{NDM-1} has spread

across the globe [9]. Studies on CRKP surveillance in Vietnam have reported bla_{NDM-1} among isolates from various sources, including patients, healthy volunteers and the environment, indicating an increasing prevalence of this carbapenemase gene in the country [10–14].

The bla_{NDM-1} gene is commonly encoded on conjugative plasmids that can transfer between bacteria. The dynamic nature of plasmids makes it difficult to obtain useful information from traditional plasmid characterization techniques like S1-nuclease pulsed-field gel electrophoresis (PFGE) or polymerase chain reaction (PCR)-based replicon typing [15]. Furthermore, these methods
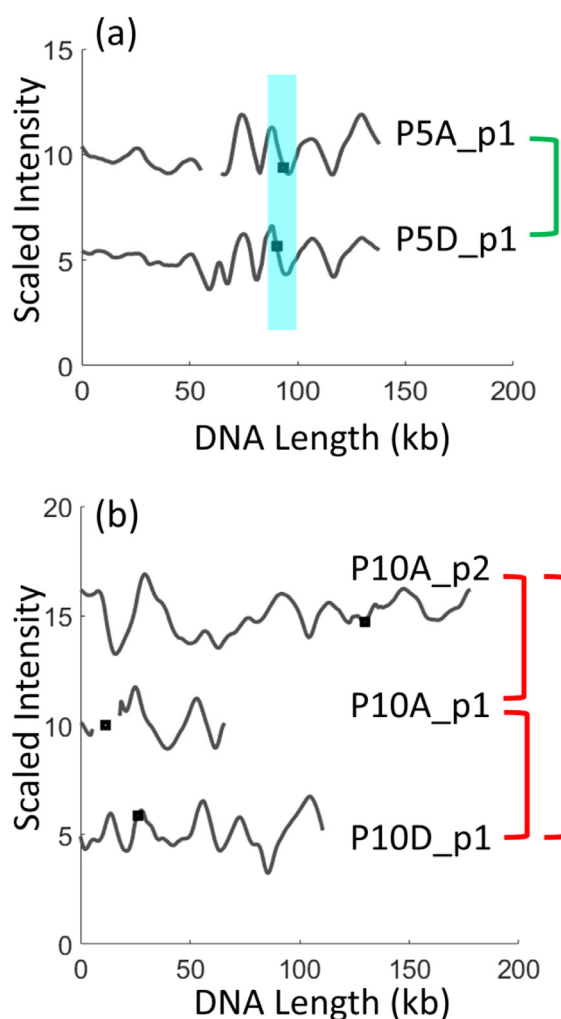
Figure 3. Barcodes for *K. pneumoniae* isolates collected from the same patient at admission and discharge. (a) Barcodes for plasmids carrying bla_{NDM-1} from P5 (ST22) at admission (P5A, one plasmid) and discharge (P5D, one plasmid). (b) Barcodes for plasmids carrying bla_{NDM-1} from P10 (ST15) at admission (P10A, two plasmids) and discharge (P10D, one plasmid). $P \leq 0.001$ is denoted by green brackets (good match) and $P \geq 0.01$ is denoted by red brackets (no match). The black square indicates the position of the bla_{NDM-1} gene.

Figure 4. Barcodes for bla_{NDM-1}-carrying plasmids from isolates of the same sequence types (STs). (a) Barcodes for plasmids carrying bla_{NDM-1} in isolates P2D, P7D and P10D (ST15). (b) Barcodes for plasmids carrying bla_{NDM-1} in isolates P3D, P10A and P9A (ST15). (c) Barcodes for plasmids carrying bla_{NDM-1} in isolates P4D and P12D (ST5015). $P \leq 0.001$ is denoted by green brackets (good match), $0.001 < P < 0.01$ is denoted by yellow brackets (partial match) and $P \geq 0.01$ is denoted by red brackets (no match). The black square indicates the position of bla_{NDM-1}, the light blue shaded region indicates that the gene is present at the same location in all plasmids, and the regions shaded in purple indicate that the gene is present at a different location.

are associated with poor resolution and are labor-intensive. Next-generation sequencing (NGS) is a widely used alternative to these traditional techniques, but requires skilled bioinformaticians to obtain the required information from plasmids and is rather expensive, thereby increasing the burden to healthcare systems in lower-to middle-income countries (LMIC). Complete assembly of plasmid sequences usually requires long-read sequencing, which is still not standard in microbiology labs.

Optical DNA mapping (ODM) is an emerging technology that is based on visualizing the sequence of large DNA molecules [16]. We developed an ODM assay that creates "barcodes" on single DNA molecules and demonstrated that it is highly suitable for imaging of intact bacterial plasmids [17,18]. The barcodes can be used to characterize bacterial plasmids and compare plasmids between samples and, in combination with CRISPR/Cas9, these barcodes provide information about the location of plasmid-borne resistance genes [4]. The barcode pattern can be predicted from the sequence of plasmids; therefore, it is possible to correlate the barcode to the presence/absence of specific genes or to find already characterized plasmids in sequence databases [17,19]. The effectiveness of
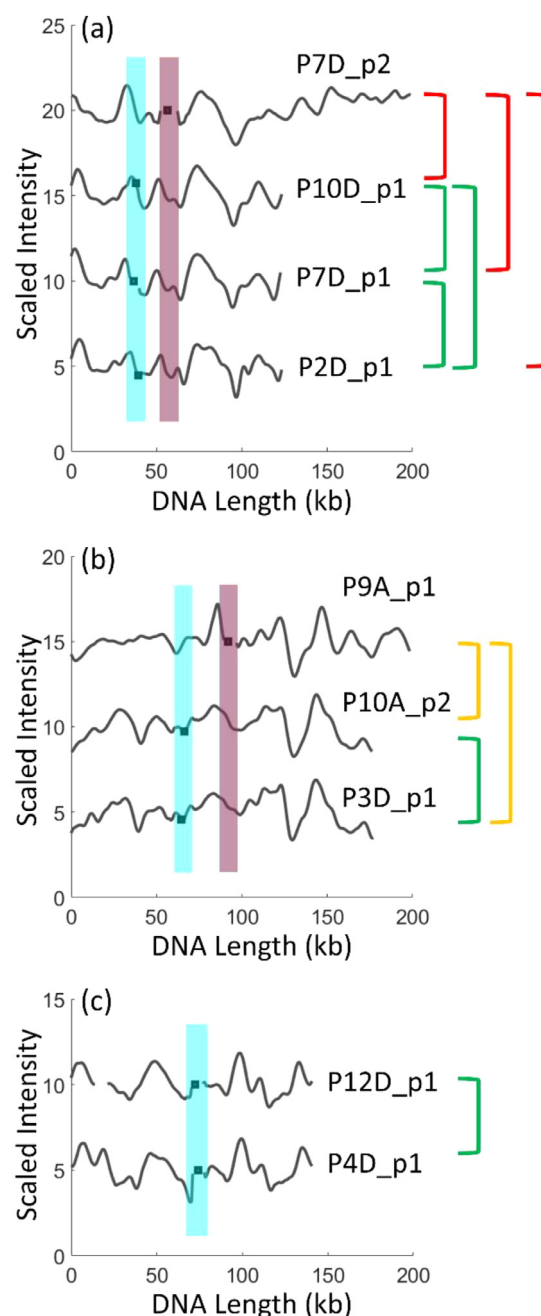
ODM for plasmid characterization has been established by studying different types of ESBL-producing Enterobacteriaceae in several hospital studies [18–21]. In these studies, the assay has been benchmarked to routine techniques, particularly different sequencing methodologies. In this study, bla_{NDM-1}-encoding plasmids from 18 isolates collected from 16 patients during a screening effort at a large Vietnamese pediatric hospital were analysed and compared using ODM.
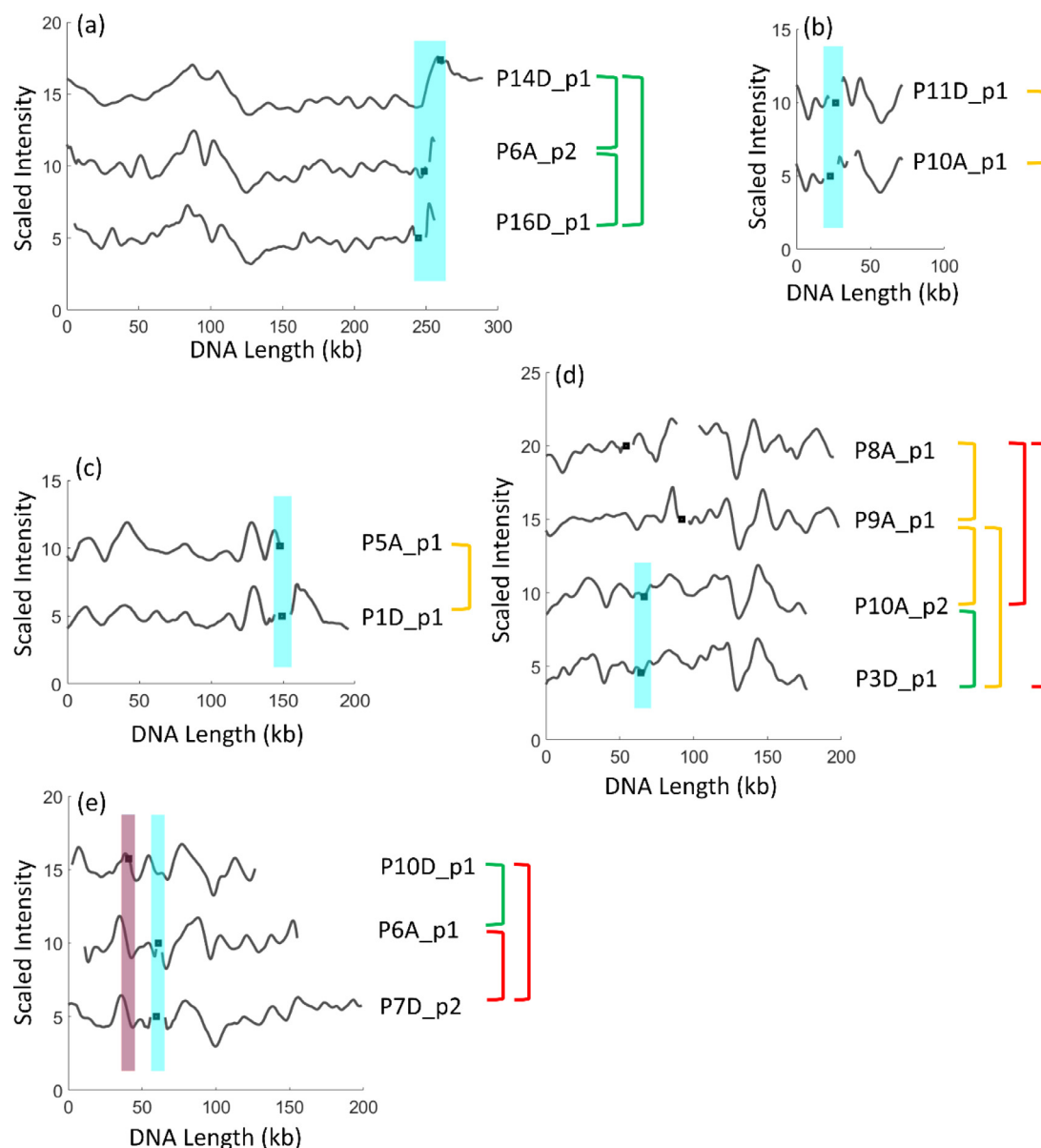
Figure 5. Barcodes for similar plasmids carrying bla_{NDM-1} in isolates of different sequence types (STs). (a) Barcodes for isolates P16D (P16D_p1: 272 kb, ST5016), P14D (P14D_p1: 289 kb, ST3003) and P6A (P6A_p2: 266 kb, ST5017). (b) Barcodes for isolates P11D (P11D_p1: 72 kb, ST5014) and P10A (P10A_p1: 66 kb, ST15). (c) Barcodes for isolates P1D (P1D_p1: 195 kb, ST1043) and P5A (P5A_p: 136 kb, ST22). (d) Barcodes for isolates P3D (P3D_p1: 163 kb, ST15), P10A (P10A_p2: 162 kb, ST15), P9A (P9A_p1: 198 kb, ST15) and P8A (P8A_p1: 197 kb, ST3228). (e) Barcodes for isolates P10D (P10D_p1: 113 kb, ST15), P6A (P6A_p1: 135 kb, ST5017) and P7D (P7D_p2: 199 kb, ST15). $P \le 0.001$ is denoted by green brackets (good match), $0.001 < P < 0.01$ is denoted by yellow brackets (partial match) and $P \ge 0.01$ is denoted by red brackets (no match). The black square indicates the position of bla_{NDM-1}, the light blue shaded regions indicate that the gene is present at the same location and the region shaded in purple indicates that the gene is present at a different location.

2. Materials and Methods

2.1. Bacterial isolates

Fecal samples were collected from 326 neonates using rectal swabs at admission and discharge during a previous study performed at a large Vietnamese pediatric hospital [8]. Samples were screened for carbapenem-resistant *K. pneumoniae* on CarbaID agar (bioMérieux, Marcy l'Etoile, France) and species-determined with the VITEK 2 system (bioMérieux). Of 149 whole-genome sequenced isolates (see below), a total of 18 isolates from 16 neonates positive for bla_{NDM-1} were selected for ODM analysis [5]. Among them, two isolates of *K. pneumoniae* available from samples taken from patients at both admission and discharge were included.

2.2. Next-generation sequencing and typing

First, total DNA was extracted using the EZ1 DNA Tissue Kit and the EZ1 Advanced XL instrument (Qiagen, Hilden, Germany). DNA concentration was determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) and 20 ng from each isolate was used for sequence library construction using a QIAseq FX DNA Library Kit (Qiagen). The Qubit 2.0 Fluorometer and QI-Axcel instrument (Qiagen) were used to measure the quantity and quality of the DNA prior to paired-end sequencing on MiSeq instrument (Illumina, San Diego, CA, USA). Raw reads were deposited at GenBank (Accession: SRR12149859-SRR12149876). The raw reads were assembled using CLC Genomics Workbench v.9.5.3 (Qiagen). Species of the isolates were verified using the k-mer-based
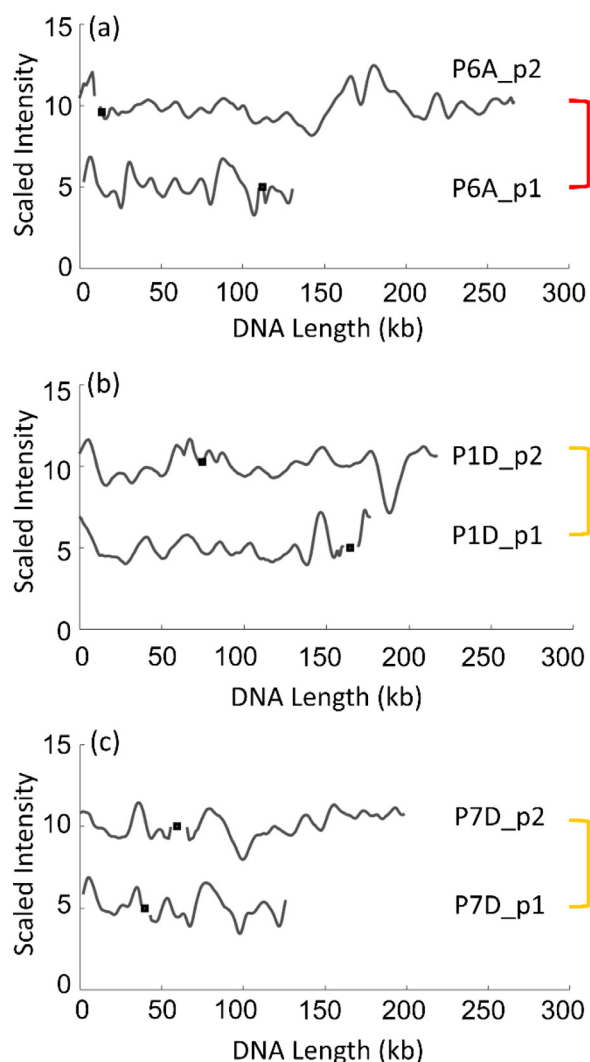
Figure 6. Barcodes comparing plasmids in isolates where more than one plasmid carrying bla_{NDM-1} was found. $0.001 < P < 0.01$ is denoted by yellow brackets (partial match) and $P \geq 0.01$ is denoted by red brackets (no match). The black square indicates the position of the bla_{NDM-1} gene.

tool in CLC Genomics Workbench. Multi-locus sequence typing (MLST) and resistome profile of bla_{NDM-1} carriage were obtained by querying the MLST typing tool version 2.0.4 and ResFinder 3.2 hosted at the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services). Unknown sequence types (STs) were assigned new STs through the Institut Pasteur MLST and whole-genome MLST databases. Plasmid replicon typing was carried out with PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

2.3. Plasmid extraction

Pure plasmids were extracted using the NucleoBond® Xtra Midi (Macherey-Nagel) plasmid purification kit following manufacturer's instructions. This kit is suitable for purification of different size plasmids ranging from 3 kbp to 300 kbp and is associated with a very good yield. The plasmid concentration was measured using the Qubit^{TM} dsDNA BR Assay (ThermoFisher).

2.4. Optical DNA mapping (ODM)

The ODM assay for plasmid characterization used in this study has been well documented [4,18-20]. A schematic of the assay

is shown in Figure 1. Plasmids extracted from isolates from the 16 patients were treated with Cas9 targeting the bla_{NDM-1} gene and labeled with YOYO-1, a non-specific fluorescent dye, and netropsin, a ligand that specifically binds to AT-rich regions of double-stranded DNA to form a "DNA barcode". The plasmids were then stretched in nanochannels and the barcodes that were used for analysis were obtained. Details can be found in the Supporting Information.

Data analysis: Details on image analysis and statistical methods used in this study has been discussed in detail in earlier studies [4,17]. The lengths of the bla_{NDM-1} plasmids were obtained using λ-DNA as reference [22], The barcodes were used to compare plasmids encoding bla_{NDM-1} between isolates to assess possible plasmid transfer. A statistical test was used to generate P-values that were in turn used to classify plasmid pairs based on their similarity [18]. $P \leq 0.001$ indicates a "good match" and is represented by a green box in Figure 2, meaning that the plasmids were identical within the resolution of the measurement, potentially with an insertion or deletion in one of them. $0.001 < P < 0.01$ indicates a "partial match" and is represented by a yellow box in Figure 2. This was observed when the barcodes matched well in overlapping regions, but also had regions that did not match. $P \geq 0.01$ indicates "no match" and is represented by a red box in Figure 2.

To compare sequences obtained from NGS to the ODM barcodes, the NGS contigs were first converted into barcodes using a custom-written Matlab code [23,24]. The contig barcodes were then matched against the corresponding ODM barcode and a score was calculated to estimate the match between the two barcodes.

3. Results

MLST results are summarized together with additional information in Table S1 (Supporting Information). Species-determination based on k-mers of the sequence reads showed that 3/18 isolates belonged to *Klebsiella quasipneumoniae*, a recently identified new species that is challenging to distinguish from *K. pneumoniae* through standard clinical laboratory testing [23], and is considered a part of *K. pneumoniae* sensu latu. ODM was performed on plasmids from all 18 isolates, and a total of 22 plasmids were identified to be carrying the bla_{NDM-1} gene (four of the 18 isolates carried two plasmids with bla_{NDM-1}). Figure 2a shows a histogram of the sizes of all the plasmids in which bla_{NDM-1} was identified. The plasmids are named based on patient number (1-16) and whether they were collected at admission (A) or discharge (D) and then given a unique number (_1 or _2). There was a large variation in plasmid sizes between the isolates, ranging from 66 kb (P10A_p2) to 289 kb (P14D_p1).

Once the plasmids carrying the bla_{NDM-1} gene were identified in all isolates, barcodes were compared and the results summarized in a similarity matrix (Figure 2b). The plasmids were classified as a "good match" ($P \leq 0.001$, green boxes), a "partial match" ($0.001 < P < 0.01$, yellow boxes) or "no match" ($P \geq 0.01$, red boxes) (see Methods). A total of ~80% of the boxes in the matrix are "red", which indicates that there are many different plasmids carrying the bla_{NDM-1} gene. Below we further investigate the similarity matrix and discuss instances when we expect the same plasmid to be present or not present.

Admission and discharge

Isolates collected at admission (adm.) and discharge (dis.) from the same patient were expected to have the same plasmid, particularly if they belonged to the same ST. Pairs of isolates from admission and discharge were available for two patients. Isolates P5A (adm.) and P5D (dis.) from patient 5 both belonged to ST22. P5A had a 136 kb plasmid (P5A_p1) and isolate P5D had a 138 kb plasmid (P5D_p1) carrying the bla_{NDM-1}gene. The P-value for the comparison of the barcodes of these plasmids was below 0.001,

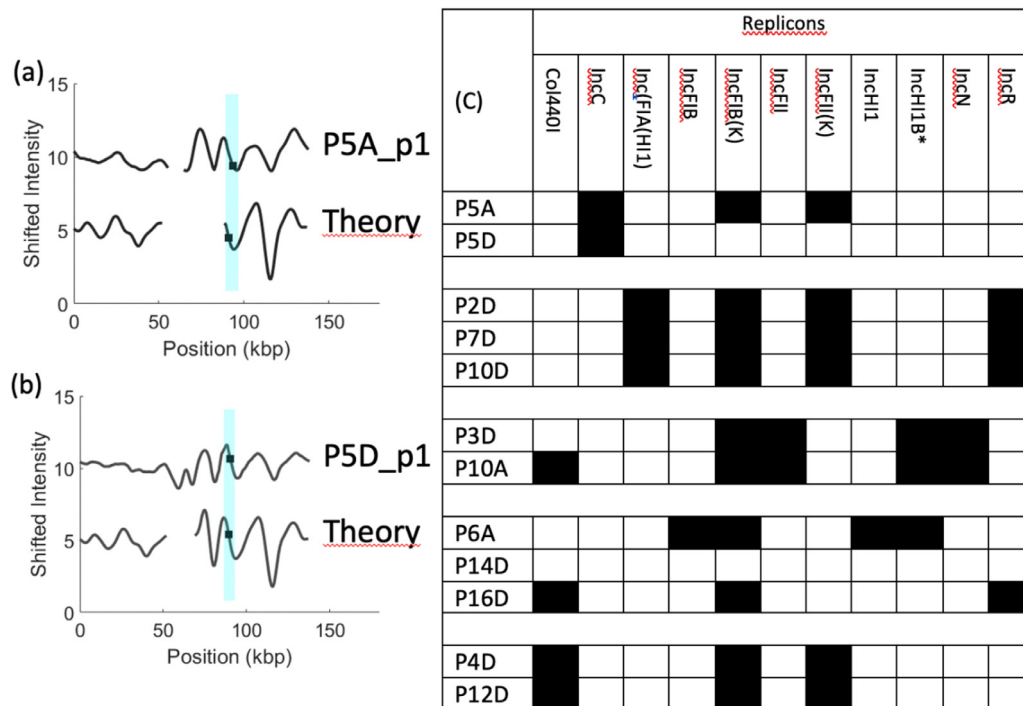| (C)	Col440I	IncC	IncFIA(HI1)	IncFIB	IncFIB(K)	IncFII	IncFII(K)	IncHI1	IncHI1B*	IncN	IncR
P5A		■			■		■				
P5D		■									
P2D			■		■		■		■		■
P7D			■		■		■		■		■
P10D			■		■		■		■		■
P3D					■		■		■		
P10A	■				■		■		■		
P6A				■			■	■			
P14D							■				
P16D	■				■						■
P4D	■				■						
P12D	■				■						

Figure 7. (a) Experimental barcode (black) and theoretically predicted barcode (gray) for plasmid P5A_p1. (b) As in (a), but for plasmid P5D_p1. The black square indicates the position of bla_{NDM-1} and the shaded region indicates that the gene is present at the same location in the experimental barcode and the barcode predicted from the sequence. (c) Black squares indicate replicons found in the sequence data obtained with NGS in pairs or trios where ODM indicates that the plasmids are indistinguishable. *=(pNDM-MAR)

meaning that it was a good match and that the plasmid was indistinguishable between the two isolates (Figure 3a).

Isolates P10A (adm.) and P10D (dis.) were collected from patient 10, and both belonged to ST15. Isolate P10A contained two different plasmids carrying bla_{NDM-1} and P10D contained one. Interestingly, the comparison with ODM revealed that the plasmids carrying the bla_{NDM-1} gene differed between the time of admission (P10A_p1: 66 kb; P10A_p2: 162 kb) and the time of discharge (P10D_p1: 113 kb) in patient 10 (Figure 3b).

Comparison between plasmids within the same sequence types

Earlier studies have shown that CRKP belonging to the emerging multidrug-resistant, high-risk clone ST15 have been spreading in hospital settings in Vietnam [13,25]. Six *K. pneumoniae* isolates in this study belonged to ST15. Among the six isolates, two were collected at admission (P9A and P10A) and four at discharge (P2D, P3D, P7D and P10D) from a total of five different patients. As discussed above, P10A and P10D were collected from the same patient (patient 10) at the time of admission and discharge, but the plasmids were not the same. P7D and P10A contained two plasmids each with bla_{NDM-1} (named P7D_p1, P7D_p2, P10A_p1 and P10A_p2, respectively).

An identical plasmid ($P < 0.001$) of 113 kb was identified in isolates P2D, P7D and P10D and had the bla_{NDM-1} gene at the same location along the plasmid contour (Figure 4a). These three isolates were all collected from samples taken at discharge and a possible explanation for their similarity is nosocomial strain transmission [21].

Interestingly, the second plasmid found in isolate P7D of 199 kb (P7D_p2) was ~85 kb longer than plasmids P2D_p1, P7D_p1 and P10D_p1, all of which belong to ST15, and showed a similar barcode but the gene was located at a different site compared to the other three plasmids.

Furthermore, an identical ~162 kb plasmid was found in isolates P3D and P10A, with an identical bla_{NDM-1} gene location

(Figure 4b). Importantly, plasmid P10A_p1 was present in the isolate collected at admission and plasmid P3D_p1 at discharge, indicating possible bacterial transmission in the hospital environment. P9A, belonging to ST15, had a 198 kb plasmid and its barcode was very similar to plasmids P3D_p1 (ST15) and P10A_p2 (ST15); however, the bla_{NDM-1} gene was at a different location (Figure 4b). This again indicated a potential common origin of the two plasmids. P10A_1 was short (66 kb) and was unique among the ST15 isolates.

Isolates P4D and P12D originated from different patients but both were identified as *K. quasipneumoniae* ST5015. The isolates carried plasmids P12D_p1 and P4D_p1 with highly identical barcodes and the bla_{NDM-1} gene at the same location; however, P12D_p1 (147 kb) had a 6 kb insertion compared with P4D_p1 (141 kb).

Comparison between plasmids across different sequence types

To investigate potential plasmid conjugation, plasmids from isolates of different STs were compared (Figure 5). In five cases, similar plasmids were found in different STs. The *K. quasipneumoniae* isolate, P16D with plasmid P16D_p1 (272 kb, ST5016), and the two *K. pneumoniae* isolates, P6A with plasmid P6A_p2 (266 kb, ST5017) and P14D with plasmid P14D_p1 (289 kb, ST3003), showed barcodes with a good match ($P < 0.001$) (Figure 5a), and a similar location for bla_{NDM-1}, although P14D_p1 was ~20 kb longer than the other two plasmids.

Plasmid P10A_p1 (66 kb, ST15) showed a good match with plasmid P11D_p1 (72 kb, ST5014) and was of similar length (Figure 5b). Among other STs, there was a match between plasmid P1D_p1 (195 kb, ST1043) and plasmid P5A_p1 (136 kb, ST22), with identical barcodes and bla_{NDM-1} gene location (Figure 5c), but the P5A_p1 plasmid was ~60 kb shorter. Plasmid P8A_p1 (197 kb, ST 3228) had a similar barcode to that of plasmids P10A_p2 (162 kb), P3D_p1 (163 kb) and P9A_p1 (198 kb) of ST15 (Figure 5d); P3D_p1 and P10A_p2 had identical plasmids (Figure 4b). The best match was with plasmid P9A_p1 (yellow), but the bla_{NDM-1} gene location differed, in-

dicating that the plasmids were not identical. P10D_p1 (113 kb, ST15) matched well ($P \leq 0.001$) with P6A_p1 (135 kb, ST5017), but had a different gene location (Figure 5e). On the other hand, some regions of the barcode for P7D_p2 (199 kb, ST15) were identical to both P10D_p1 and P6A_p1 and had a similar gene location as P6A_p1; however, these plasmids were of different lengths (∼60 kb).

In addition to these matches, four isolates carried unique plasmids containing bla_{NDM-1}. Isolate P1D (Patient 1, ST1043) had two plasmids (Figure 6a) of lengths 195 kb (P1D_p1) and 217 kb (P1D_p2) of which P1D_p2 did not match with any plasmids at all. Similarly, isolate P13A (Patient 13, ST17) carried the bla_{NDM-1} gene in a 106 kb plasmid and isolate P15D carried bla_{NDM-1} in a unique 154 kb plasmid. Other plasmids showed partial matches for barcodes, P9A_p1 (198 kb, Patient 9, ST15) and P8A_p1 (197 kb, Patient 8, ST 3228), but they had different gene locations.

Isolates with more than one plasmid carrying *bla* **NDM-1**

Interestingly, of the 18 isolates studied, four contained two plasmids carrying the bla_{NDM-1} gene. Methods traditionally used to identify resistance genes in a sample, such as PCR, cannot identify whether the same gene is present in two plasmids in the same isolate, but the ODM methodology clearly identifies all plasmids in an isolate carrying a specific gene (Figure 3B, Figure 6). For P10A, P10A_p1 (66 kb) and P10A_p2 (162 kb) are non-identical plasmids in terms of length, barcode, and gene location. Furthermore, P6A_p1 (135 kb) was not similar to P6A_p2 (266 kb) (Figure 6a) and P1D_p1 (195 kb) was different to P1D_p2 (217 kb) (Figure 6b). However, for isolate P7D, P7D_p1 (113 kb) and P7D_p2 (199 kb) had identical barcodes for the overlapping regions although the sizes differed (Figure 6c). An experiment was conducted without cutting the circular plasmids with Cas9 for isolate P7D, with the goal of confirming that P7D_p1 and P7D_p2 were indeed intact plasmids. This isolate was found to have plasmids of three different sizes ∼74 kb, ∼115 kb and ∼190 kb, of which the latter two were the ones that carried bla_{NDM-1}. The two shorter plasmids combined are the size of the longer plasmid, indicating that the P7D_p2 plasmid is a combination of the two smaller plasmids. Such structural variations are common for plasmids. From the experiments with circular plasmids, the two shorter plasmids were noted to be much more common than the longest plasmid. More details on this experiment can be found in the Supplementary Information.

Comparison of ODM and NGS data

To corroborate the findings obtained with the ODM methodology, the NGS data were compared with the ODM data in two different ways. First, the NGS data were used to identify contigs containing bla_{NDM-1} that were long enough to enable matching to the ODM data (Figure 7A-B) [24]. One such contig was found in isolate P5A and one in P5D. As it is possible to predict barcodes from sequences [17,23], these contigs can be matched to the experimental barcodes. Although the contigs did not cover the entire plasmids for either of the isolates, a very good match was observed in the region where it did cover. Furthermore, the position of bla_{NDM-1} was the same in both the ODM experiment and on the contig obtained through NGS.

Second, we used PlasmidFinder (see Methods) to compare the NGS data with the ODM data (Table S2). We compared the plasmids replicons for the five cases with indistinguishable ODM patterns (Figure 7C). In four cases the replicon content was identical or very similar, differing only in the presence of only one replicon, while for the fifth case (P6A, P14D and P16D), there was no similarity in the identified plasmid replicons, suggesting that the bla_{NDM-1}-plasmids in these isolates could have a replicon that is not included in the typing scheme.

Discussion

A total of 22 plasmids carrying the bla_{NDM-1} gene were identified in 18 fecal isolates from 16 neonates admitted to a Vietnamese hospital; four isolates carried two different bla_{NDM-1}-encoding plasmids. Among the 22 plasmids, there were more than 10 different types of plasmids with sizes ranging from 66 kb to 289 kb. This demonstrates that many plasmids carrying bla_{NDM-1} were present in this hospital cohort and that not just a few successful plasmids were responsible for the rapid spread of bla_{NDM-1} in the investigated population.

Although there was large plasmid diversity, there was potential evidence for both clonal spread and plasmid transmission in the hospital environment. Two cases indicated strain transmission and three other cases indicated plasmid conjugation. One of the three cases indicated plasmid conjugation between *K. pneumoniae* and *K. quasipneumoniae*. Notably, ODM analysis does not determine when transmission and conjugation occurred.

Analysis of plasmid barcodes from *K. pneumoniae* isolated from samples collected at both admission and discharge from two different patients demonstrated in one case that the plasmids at discharge differed from those at admission. This indicates that either the gene translocated to another plasmid, the strain acquired a new plasmid during the patient's stay in hospital, or the dominant strain at discharge was only present to a small extent at admission or *vice versa*, and that one strain was thus potentially overlooked at one of the sampling occasions.

The ODM methodology has an important technical advantage for this type of analysis. Isolates from four of the patients were found to carry two different plasmids with bla_{NDM-1}. This observation is possible with the ODM technique because it is based on analyses of single molecules separately. In contrast, analysis with more commonly used PCR-based methods would fail to distinguish between the different plasmids. It is unclear how common this phenomenon is because of the limitations of existing techniques. A *K. pneumoniae* strain harboring two plasmids with bla_{NDM-1} has been discussed in an earlier study based on genome sequencing [26]. Plasmid pairs were also observed for which regions of the plasmids were very similar, but there were larger structural variations.

The ODM method for plasmid analysis has been established and evaluated in several studies [4,17-21]. For example, results from ODM have been validated by comparing the results with long-read sequencing data in several studies [19,21,27]. In the current study, NGS was used to select the samples investigated and the ODM results were compared with the NGS data. For two isolates, long contigs (121 kb and 105 kb, respectively) containing bla_{NDM-1} were obtained and the overlap between barcodes predicted from the NGS data and the ODM barcodes was very good. There was also good agreement for the overlap between plasmid genes identified with PlasmidFinder and the observations with ODM. The one case where there was disagreement between the methods may be due to the bla_{NDM-1}-carrying plasmid having a replicon not available in PlasmidFinder. Importantly, the ODM method is independent of what is included in the databases used, as no sequence specific probes are used.

The lack of user-friendly and inexpensive tools for studying plasmid transmission is a major shortcoming when addressing potential plasmid transmission in hospitals. Thus, the ODM method holds great promise for complementing current infection control tools and could represent a means to track in-hospital transmission, including in resource-poor settings.

Competing Interests

None to declare.

Ethical Approval

Ethics approval for this study was granted by the Ethics Committee of the Vietnam National Children's Hospital (reference number: VNCH-RICH-16-014 with approved extension/appendix).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2021.106496.

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