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Rapid Detection of New Delhi Metallo-β-Lactamase Gene and Variants Coding for Carbapenemases with Different Activities by Use of a PCR-Based *In Vitro* Protein Expression Method

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New Delhi metallo-β-lactamase (NDM)-producing bacteria are considered potential global health threats. It is necessary to monitor NDM-1 and its variants in clinical isolates in order to understand the NDM-1 epidemic and the impact of its variants on β-lactam resistance. To reduce the lengthy time needed for cloning and expression of NDM-1 variants, a novel PCR-based *in vitro* protein expression (PCR-P) method was used to detect bla_{NDM-1} and its variants coding for carbapenemases with different activities (functional variants). The PCR-P method combined a long-fragment real-time quantitative PCR (LF-qPCR) with *in vitro* cell-free expression to convert the bla_{NDM-1} amplicons into NDM for carbapenemase assay. The method could screen for bla_{NDM-1} within 3 h with a detection limit of 5 copies and identify functional variants within 1 day. Using the PCR-P to analyze 5 recent bla_{NDM-1} variants, 2 functional variants, bla_{NDM-4} and bla_{NDM-5}, were revealed. In the initial testing of 23 clinical isolates, the PCR-P assay correctly found 8 isolates containing bla_{NDM-1}. This novel method provides the first integrated approach for rapidly detecting the full-length bla_{NDM-1} and revealing its functional variants in clinical isolates.

Limited antibiotic choices are available for treating patients infected with *Enterobacteriaceae*, *Acinetobacter* spp., and other species producing New Delhi metallo-β-lactamase 1 (NDM-1; encoded by the bla_{NDM-1} gene, nucleotide sequence accession no. KF016990.1) because they are usually resistant to all classes of antibiotics, including carbapenems (1). Much worse, bla_{NDM-1} is commonly found on plasmids, which could easily transfer between species (2, 3). To date, various kinds of species containing this gene have been isolated and found worldwide (4–6). Therefore, the infections caused by NDM-1-producing bacteria are considered potential global health problems (1). At the same time, along with its spread, variants of NDM-1 are emerging. Until now, 10 NDM variants have been found (7–13; also NDM-9 [nucleotide sequence no. KC999080.1] and NDM-10 [nucleotide sequence accession no. KF361506.1]). Some of the variants, such as NDM-4 and NDM-5, confer higher β-lactam resistance to bacteria than NDM-1 because they pose increased carbapenemase activities compared to that of wild-type NDM-1 (9, 10). In order to better understand the NDM-1 epidemic and the impact of the NDM variants on β-lactam resistance, it is necessary to monitor NDM-1 and its variants in clinical isolates.

Phenotypic techniques for detecting carbapenemase activity, such as the modified Hodge Test (MHT) recommended by the Clinical and Laboratory Standards Institute (CLSI) (14, 15) and the newly developed Carba NP (16), can be used to detect carbapenemase producers, including those producing NDM-1. However, because all of the phenotypic assays detect NDM-1 together with other carbapenemases, specific molecular methods to detect the bla_{NDM-1} gene have been widely used to indicate the presence of NDM-1-producing bacteria (17–21). These methods share the feature that only a small fragment of the bla_{NDM-1} gene is PCR amplified, and as a consequence, they can only report the presence of bla_{NDM-1}. To obtain mutation information, PCR amplification of the full-length bla_{NDM-1} plus DNA sequencing are normally used (11). DNA sequencing can reveal every possible mutation of bla_{NDM-1} but cannot give function information as to whether the mutations will promote or reduce the ability of the coded NDM to hydrolyze β-lactam (9). For example, the amino acid substitution in NDM-4 is not located in the known active sites of NDM-1, indicating that remote amino acid substitutions might also play a role in the extended activity of NDM-4 (9). Expression and purification of the recombinant NDM are normally used to study the effects of the new mutations, but the process is time consuming (from a few days to weeks).

In this study, a new strategy called PCR-based *in vitro* protein expression (PCR-P) is explored for rapid detection of the full-length bla_{NDM-1} and its variants coding for carbapenemases with different activities (functional variants). The PCR-P method consists of a novel long-fragment quantitative PCR (LF-qPCR) to detect the full-length bla_{NDM-1} in clinical isolates and a function assay of *in vitro*-synthesized protein to identify whether the LF-qPCR amplicons contain bla_{NDM-1} or its functional variants through measuring the imipenem degradation rate of the *in vitro*-synthesized NDM. As far as we know, this is the first assay to

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TABLE 1 Sample information and results of phenotypic assay, conventional PCR, MLST, LF-qPCR, *in vitro*-synthesized carbapenemase activity assay, and sequencing[a]

Strain or variant	Vitek 2 MIC (mg/liter) of:				Result of indicated step of PCR-P assay		
	IPM	ETP[b]	PCR[c]	ST[d]	LF-qPCR (C_T value)	Antibiotic degradation assay (rate [$-\%A_0$/min])[e]	Sequencing result(s)
Strains							
E. coli 53 (negative control)	≤1	2	Neg	—	Neg	—	—
A. baumannii 65 (positive control)	≥16	—	+	New	13.27	1.338 ± 0.135	WT
E. coli 23	≤1	2	Neg	—	Neg	—	—
E. coli 59	2	≤0.5	Neg	—	Neg	—	—
E. coli 75	≤1	2	Pos	—	12.89	1.393 ± 0.083	WT
K. pneumoniae 17	≥16	≥8	Pos	—	28.24	1.344 ± 0.092	WT
K. pneumoniae 19	2	1	Neg	—	Neg	—	—
K. pneumoniae 76	2	1	Neg	—	Neg	—	—
E. cloacae 60	≤1	4	Neg	—	Neg	—	—
E. cloacae 63	2	≤0.5	Neg	—	Neg	—	—
K. ozaenae 16	12	≥8	Pos	—	14.4	1.264 ± 0.134	WT
A. baumannii 26	≥16	—	Neg	254	Neg	—	—
A. baumannii 30	≥16	—	Neg	254	Neg	—	—
A. baumannii 31	≥16	—	Pos	254	30.96	1.284 ± 0.093	WT
A. baumannii 33	≥16	—	Neg	254	Neg	—	—
A. baumannii 39	≥16	—	Neg	254	Neg	—	—
A. baumannii 40	≥16	—	Neg	254	Neg	—	—
A. baumannii 41	≥16	—	Pos	254	28	1.301 ± 0.089	WT
A. baumannii 42	≥16	—	Neg	New	Neg	—	—
A. baumannii 59	8	—	Neg	208	Neg	—	—
A. baumannii 62	≥16	—	Pos	208	24.94	1.181 ± 0.245	WT
A. baumannii 63	≥16	—	Pos	381	26.58	1.391 ± 0.053	WT
A. baumannii 66	≥16	—	Neg	368	Neg	—	—
A. baumannii 69	≥16	—	Neg	New	Neg	—	—
A. baumannii 70	≥16	—	Pos	229	14.23	1.511 ± 0.087	WT
Variants[f]							
NDM-K211E	—	—	Pos	—	10.63	0.132 ± 0.004	A611G
NDM-2(P28A)	—	—	Pos	—	15.73	1.140 ± 0.069	C82G
NDM-3(D95N)	—	—	Pos	—	11.52	1.120 ± 0.047	G283A
NDM-4(M154L)	—	—	Pos	—	11.30	2.236 ± 0.060	A460C
NDM-5(V88L, M154L)	—	—	Pos	—	12.63	1.773 ± 0.017	G262T, A460C
NDM-6(A233V)	—	—	Pos	—	13.27	1.187 ± 0.105	C698T

[a] IPM, imipenem; ETP, ertapenem; ST, sequence type; Pos, positive; Neg, negative; —, not tested; WT, wild type.
[b] Ertapenem has limited activity against *A. baumannii*.
[c] Conventional PCR to detect bla_{NDM-1}.
[d] New, new ST.
[e] $-\%A_0$/min is the percentage of absorbance decrease per minute. $\%A_0$ is the percentage of absorbance relative to the absorbance at time zero (just after imipenem is added).
[f] bla_{NDM-1} variants were constructed by using a site-directed gene mutagenesis kit and overlap PCR. The mutations in these variants were confirmed by sequencing as shown in the last column.

integrate the detection of the full-length bla_{NDM-1} with the identification of bla_{NDM-1} functional variants.

MATERIALS AND METHODS

Bacterial strains. One NDM- producing *Acinetobacter baumannii* strain (sample no. *A. baumannii* 65) isolated previously by us (22, 23), along with a non-NDM-1 producer (*Escherichia coli* 53) as the negative control, were used to optimize the novel PCR-P. As shown in Table 1 and Table S1 in the supplemental material, a collection of 23 carbapenem-resistant strains isolated from different patients in different years by the microbiology laboratory of Guangzhou First Municipal People's Hospital were chosen for testing. The MICs were determined using the Vitek 2 system (BioMerieux, France) and interpreted according to CLSI 2012 guidelines (24). Because the first NDM-1 producer reported in China was *A. baumannii* (5), *A. baumannii* isolates were mainly chosen for testing. The results of multilocus sequence typing (MLST) show that the 15 *A. baumannii* isolates belonged to 5 different known sequence types (STs) and 3 new STs (Table 1) (25). Details are given in Table S2 in the supplemental material.

A site-directed gene mutagenesis kit (Beyotime Institute of Biotechnology, Shanghai) and overlap PCR were used to create 6 bla_{NDM-1} variants, bla_{NDM-2} (GenBank accession no. JN112341), bla_{NDM-3} (JQ734687), bla_{NDM-4} (JQ348841), bla_{NDM-5} (JN104597), bla_{NDM-6} (JQ235754), and $bla_{NDM-K211E}$ (encoding a K-to-E change at position 211), using the primers shown in Table S3 in the supplemental material.

Preparation of DNA templates of clinical isolates. Both plasmid extracts and genomic DNA were used as the templates for PCR-P. Plasmids were extracted from fresh overnight bacterial cultures with an E.Z.N.A. plasmid minikit (Omega Bio-Tek, United States) according to the protocol suggested by the manufacturer. DNA templates were also obtained by

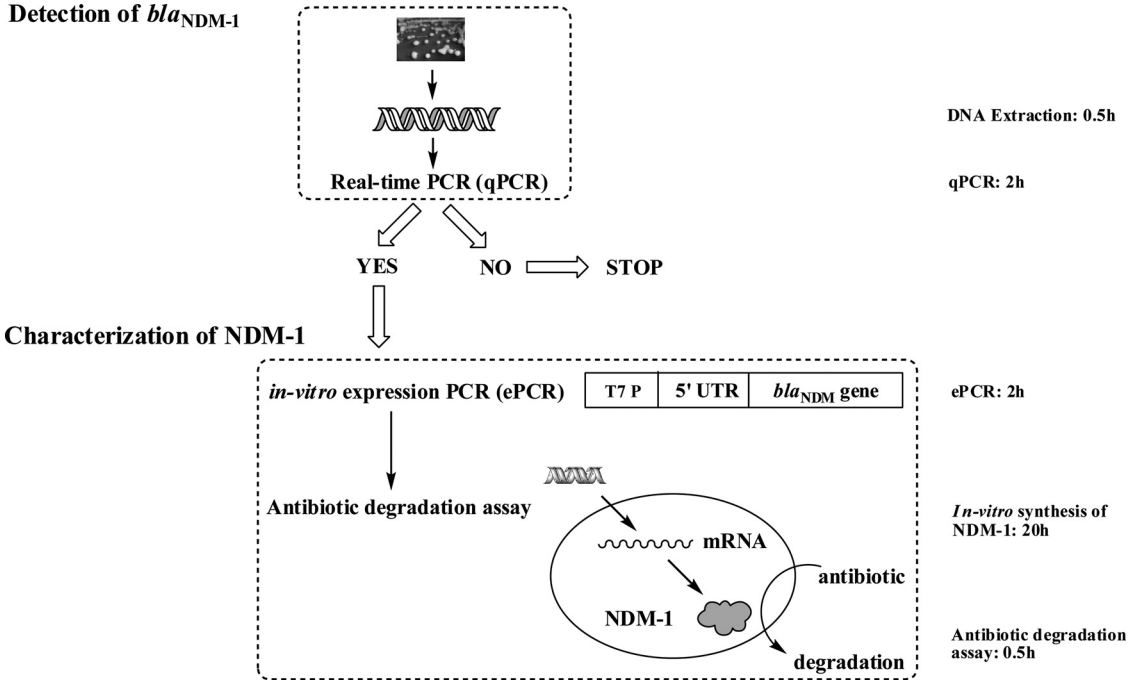

FIG 1 Scheme of the gene-to-protein function assay for rapid identification of bla_{NDM-1} and functional variants with mutations related to carbapenemase activity.

heat lysis (26, 27). Briefly, 1.4 ml bacterial culture (with a McFarland standard of between 3 to 4) was centrifuged, and then the precipitates were resuspended in 0.1 ml sterile MilliQ water and inactivated at 100°C for 10 min.

PCR-P assay procedure. As shown in Fig. 1, the first step of the method was LF-qPCR detection of bla_{NDM-1} from samples**.** The major difference of the LF-qPCR from other qPCR methods was that the full-length bla_{NDM-1} (822 bp) was amplified with the specially designed primers F-rt and R-rt (see Table S3 in the supplemental material). When a positive amplification was detected, the second step was initiated to further confirm the LF-qPCR products and reveal possible functional mutated bla_{NDM-1} variants. During this step, a second *in vitro* expression PCR (ePCR) was performed to introduce the expression elements T7 promoter and 5′ untranslated region (5′ UTR), using the primers F-1-UTR and R-1, shown in Table S3. Then, *in vitro* synthesis of NDM-1 was carried out at 24°C for 20 h using an RTS 100 wheat germ CECF kit (rapid translation system continuous-exchange cell-free kit; 5 Prime, Inc., United States). An equal amount of the lysate without the ePCR amplicon was also incubated simultaneously, as a negative control. Measurements of NDM-1 activities were performed on a Synergy H1 hybrid reader (BioTek, United States) by observing the decrease in optical density at 300 nm (OD_{300}) at 30°C, as described previously (1). The antibiotic degradation rates were compared with that of NDM-1 to identify bla_{NDM-1} functional variants that possessed either increased or decreased carbapenemase activities. The testing could be finished within 3 h for screening for bla_{NDM-1} and within 24 h for identifying bla_{NDM-1} variants. Please see the materials and methods in the supplemental material for the detailed conditions.

DNA sequencing and conventional PCR method. The positive amplicons from the LF-qPCR (containing the full-length bla_{NDM-1}) were sequenced by BGI Tech, Inc. (Wuhan, China). Conventional PCR methods, described previously (5, 7), were used to test for the presence of bla_{NDM-1} in the isolates, using the primer pairs F-38 and R-344, as well as Pre-A and Pre-B, shown in Table S3 in the supplemental material.

RESULTS

LF-qPCR assay of bla_{NDM-1}. The specificity of the primers F-rt and R-rt for bla_{NDM-1} detection was first evaluated by a BLAST search of the NCBI database (www.ncbi.nlm.nih.gov). No matches to the primers were found except for the bla_{NDM-1} gene. The specificity of LF-qPCR was further checked using the genomic DNA obtained from the non-NDM-1 producer *E. coli* 53 (negative control).

The LF-qPCR amplification generated a threshold cycle (C_T) value of more than 40 for the negative control and a C_T value of around 26 for 1×10^3 copies of the plasmid DNA extracted from *A. baumannii* 65, with a single peak in the melting curve (Fig. 2A). The signal obtained for the negative control after many cycles might be due to the formation of primer dimers (with a lower melting temperature, as shown in the inset in Fig. 2A), which is common for qPCR systems based on SYBR green I. Since the LF-qPCR generated a C_T value of about 33 for 5 copies (Fig. 2B), which was comparable with the results of other PCR methods (17–19) and low enough for most clinical samples, 35 cycles was chosen as the cutoff. The standard curve, with a linear coefficient (r^2) of 0.9995 and a slope of −3.6886 (Fig. 2C), was found by analyzing serial 10-fold dilutions of the plasmid DNA ranging from 10 copies/reaction mixture volume to 1.00×10^7 copies/reaction mixture volume (Fig. 2B). The detection limit was found to be as low as 4 CFU/reaction mixture volume (or 10^3 CFU/ml), as shown in Fig. 2D, by spiking different amounts of *A. baumannii* 65 into water.

ePCR for adding the *in vitro* expression elements. In order for the wheat germ system to express protein directly from PCR products, the T7 promoter sequence must be attached to the forward primers, and some 5′ UTR sequences can be used to enhance protein expression (28, 29). Two primer pairs (F-rt-UTR/R1 and

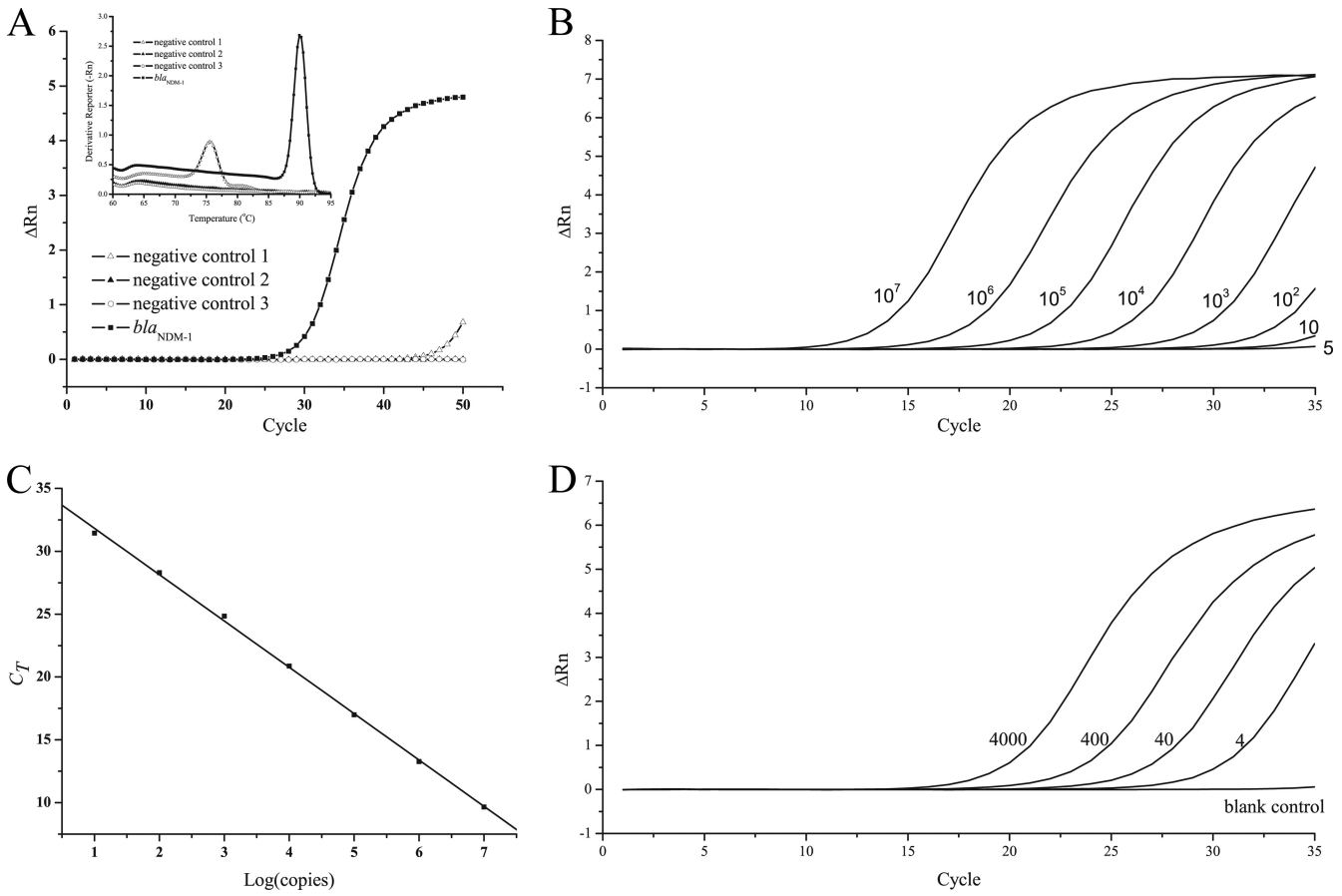

FIG 2 LF-qPCR assay of bla_{NDM-1}. (A) Amplification curves and melting curves (inset) of the negative controls and bla_{NDM-1} (10^3 copies/reaction mixture volume). (B) Amplification curves of dilutions of the plasmid (copies/reaction mixture volume) extracted from a bla_{NDM-1}-containing *A. baumannii* strain (*A. baumannii* 65). (C) Corresponding standard curve of the LF-qPCR assay. (D) Amplification curves of water samples spiked with different concentrations (CFU/reaction mixture volume) of *A. baumannii* 65. All experiments were repeated three times to determine the sensitivity and linearity of LF-qPCR. ΔRn, baseline-corrected normalized reporter.

F-1-UTR/R-1) with a 5′ UTR sequence (see Table S3 in the supplemental material) were used to amplify the bla_{NDM-1} amplicon obtained from *A. baumannii* 65 in the LF-qPCR step. The positions of the primers relative to those of the LF-qPCR primer pair are shown in Fig. S1 in the supplemental material. Both primer pairs could effectively amplify the bla_{NDM-1} gene, but there were fewer primer dimers and higher amplicon purity when using the primer pair F-1-UTR/R-1 (data not shown). Therefore, primers F-1-UTR and R-1 were used in the experiments described below. After purification, the ePCR amplicons were added into the wheat germ lysate to synthesize NDM.

Characterization of *in vitro*-synthesized NDM-1 and variants. After *in vitro* synthesis of NDM-1, the wheat germ lysates were used directly for measurement of their reactions with imipenem. As shown by the results in Fig. 3B, the OD_{300} decreased over time after imipenem was mixed with the lysate containing the bla_{NDM-1} ePCR amplicon, while no change was found for the negative-control lysate. Inhibition assays also showed that the *in vitro*-synthesized NDM-1 was strongly inhibited by EDTA. These results demonstrated that NDM-1 was successfully synthesized. Repeated experiments under the same conditions showed that the *in vitro*-synthesized-NDM assay was reproducible in terms of the imipenem degradation rate, with an intra-assay relative standard deviation (RSD) of less than 10% and an interassay RSD of less than 16%.

Using the same procedure, the degradation rates of the lysates containing different NDM variants were tested. As shown by the results in Fig. 3A, NDM-K211E degraded imipenem significantly more slowly than NDM-1, while NDM-4 and NDM-5 were found to have higher carbapenemase activities than NDM-1. Another 3 variants, NDM-2, NDM-3, and NDM-6, showed activities similar to that of NDM-1. These results were consistent with the previously reported low carbapenemase activity of NDM-K211E (30) and the higher activities of NDM-4 and NDM-5 (9, 10).

All the results described above demonstrated that the *in vitro*-synthesized NDM showed enzymatic properties similar to those of the recombinant NDM-1 and current NDM-1 variants. Therefore, it is possible to use the degradation rate of imipenem to identify the bla_{NDM-1} variants with different carbapenemase activities.

PCR-P assay of clinical isolates. As summarized in Table 1, the LF-qPCR found that 8 of the 23 clinical samples were positive for bla_{NDM-1}, which was fully consistent with the results of the conventional PCR test. The typical amplification curves for 4 positive isolates (see Fig. S2A in the supplemental material) showed that similar amplifications were obtained using either the plasmids or

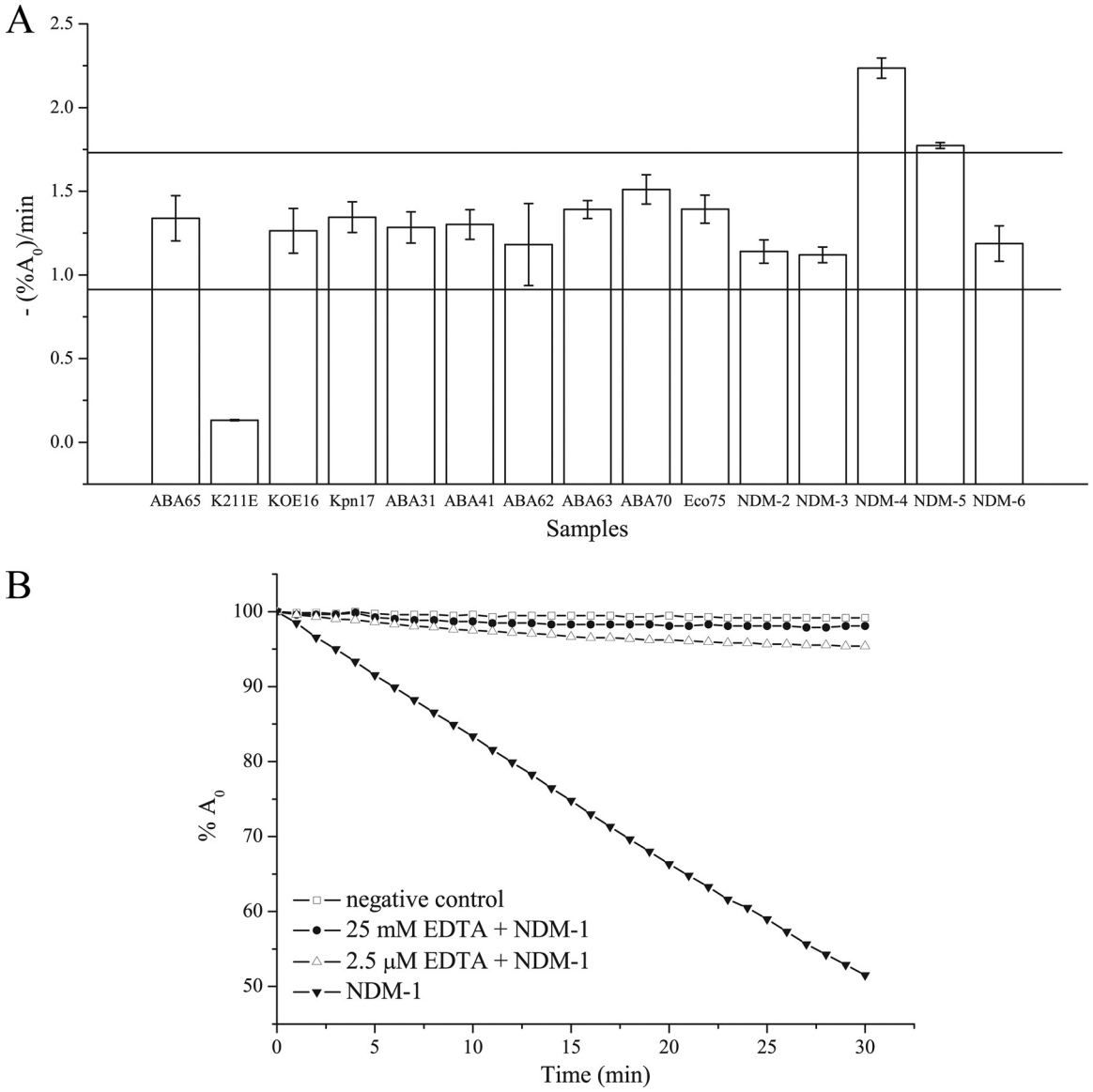

FIG 3 (A) The imipenem degradation rates of the *in vitro*-synthesized NDM variants (bla_{NDM-2}, $_{-3}$, $_{-4}$, $_{-5}$, and $_{-6}$ and $bla_{NDM-K211E}$) and the NDM-1 proteins from the positive clinical isolates in relation to the imipenem degradation rate of the wild-type positive control (*A. baumannii* strain 65). Data are the averages of three replicate experiments. Error bars show the ±SD. ABA, *A. baumannii*; KOE, *Klebsiella ozaenae*; Kpn, *Klebsiella pneumoniae*; Eco, *E. coli*. -(%A_0)/min, percentage of absorbance decrease per minute. The horizontal lines represent the cutoff values for judging functional variants, which equal the -(%A_0)/min value of the wild-type NDM-1 control plus 3 × SD (upper line) and minus 3 × SD (lower line). (B) EDTA inhibition assay for the activity of the *in vitro*-synthesized NDM-1. %A_0, percentage of absorbance relative to the absorbance at time zero (just after imipenem is added).

the templates prepared using heat lysis. All of the 8 positive isolates showed high-level resistance to imipenem by the Vitek system, except for *E. coli* sample 75. However, *E. coli* 75 was found to be resistant to ertapenem, with a MIC of 2 mg/liter (Table 1).

These LF-qPCR-positive samples were further tested with the *in vitro*-synthesized-carbapenemase assay. As shown by the results in Fig. 3A, the enzyme activities were found to be within ±3 times the standard deviation (SD) of the results for NDM-1 for all the samples. After 25 mM EDTA was added to the lysates, the imipenem degradation was inhibited (data not shown). Therefore, the PCR-P results showed that all of the 8 isolates contained bla_{NDM-1} but none contained functional variants. Fully consistent with the PCR-P results, the DNA sequencing results confirmed no mutations in the full length of the bla_{NDM-1} gene of the 8 clinical isolates.

DISCUSSION

As a mobile genetic resistance gene, bla_{NDM-1} could transfer between species and mutate under either the selection pressure of β-lactam or natural evolution during the spread. Clearly, we should pay more attention to the functional bla_{NDM-1} variants, i.e., the variants whose mutations could affect the carbapenemase activities they encode, since they can confer different levels of resistance to β-lactams. Therefore, an ideal monitoring assay should not only identify the presence of bla_{NDM-1} but also reveal functional bla_{NDM-1} variants. In

view of these requirements, the current widely used PCR sequencing method is not sufficient, because phenotypic testing (expression of recombinant protein) is needed to confirm whether a new variant identified by sequencing has an alteration in the function of its coded protein (14, 31).

Since new variants are expected to emerge due to the worldwide spread of bla_{NDM-1}, the advantage of direct identification of functional variants would be especially useful for practical applications, which is the rationale behind our work to design the PCR-P approach. As shown above, the LF-qPCR detection of the full length of bla_{NDM-1} was sensitive and fast in screening for the presence of bla_{NDM-1} in clinical isolates. The amplified long fragment not only facilitates the sensitivity of detection using SYBR green, which can reduce the cost compared to other qPCRs based on probes (17, 19), but it can also be used as the template for the *in vitro*-synthesized-carbapenemase assay for finding functional variants. Furthermore, the *in vitro*-synthesized-carbapenemase assay provides a few advantages not available by other methods. First, the positive degradation to imipenem can be used for confirmation of the LF-qPCR result. Because false amplified fragments will not result in active carbapenemase, this step could help to reduce the false-positive rate of the LF-qPCR due to nonspecific amplification. Second, functional bla_{NDM-1} variants can be revealed directly by comparing the imipenem degradation rates of the synthesized NDMs to that of NDM-1, as shown in Fig. 3A. Finally, the PCR-P assay avoids the need with the recombinant methods to study the properties of bla_{NDM-1} variants. The *in vitro* expression can be finished within 1 day, while the conventional recombinant protein procedures would take several days.

Besides bla_{NDM-1}, many other resistance-coding genes, such as the plasmid-mediated bla_{AMP-C} gene and bla_{FIM-1} gene, are monitored by PCR approaches (32, 33). Based on a similar strategy, a PCR-P assay could be developed for detecting these genes and functional variants through measuring the activity of the *in vitro*-synthesized lactamases.

However, because the PCR-P assay is basically a molecular-testing method, clinical laboratories will need molecular expertise to perform the assay. Another obstacle might be the cost, which is higher than the costs of the phenotypic methods. Therefore, we foresee that the PCR-P assay would be best utilized as a complement to a phenotypic method, such as Carba NP, for identifying bla_{NDM-1} or its functional variants.

In conclusion, with its capabilities of rapidly detecting the presence of the full-length bla_{NDM-1} gene and revealing bla_{NDM-1} functional variants, the PCR-P assay provides a unique approach for monitoring bla_{NDM-1} epidemics and functional variants in clinical isolates.

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