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Sensitive and rapid detection of bla_{NDM-1} in clinical samples by isothermal cross-priming amplification

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ABSTRACT

A highly sensitive, specific diagnostic assay for detection of bla_{NDM-1} based on cross priming amplification (CPA) was developed. The sensitivity ranged from 2.5 to 25 copies per reaction for different clinical samples. The highly and sensitive detection of bla_{NDM-1} in clinical samples highlighted the potential clinical applications of CPA.

The emergence of the New Delhi metallo-beta-lactamase 1 (NDM-1) in India, Pakistan, and the UK has sparked great attention to the threat posed by resistant bacteria (Kumarasamy et al., 2010). Bacteria that produce this carbapenemase cause infections that are difficult to treat and are therefore referred to as "superbugs". The NDM-1 was detected primarily among gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*, responsible for urinary tract infections, pneumonia and blood-stream infections (Kumarasamy et al., 2010; Chen et al., 2011). The fact that most of the isolates were from community-acquired infections suggests that the gene encoding the enzyme NDM-1 is prevalent in the environment. Therefore, the potential hazard of this new antibiotic-resistance mechanism is to be further evaluated. Molecular methods have been reported for the specific detection of bla_{NDM-1}, including DNA microarray, real time PCR and the isothermal amplification technology LAMP(Kruttgen et al., 2011; Liu et al., 2012; Naas et al., 2011). In this paper, we described a rapid and sensitive assay for the detection of NDM-1, based on the most recently developed cross priming amplification (CPA), an isothermal amplification method relies on the use of DNA polymerase of strand displacement activity (Xu et al., 2012). We evaluated its sensitivity, specificity and practical use in detection of bla_{NDM-1} in clinical samples.

For detection of bla_{NDM-1}, a set of six primers were designed based on the mechanism of CPA (6). Locations and sequences of the primers were shown in Fig. 1A. Of the six primers, F3 and B3 represented initiation primers, PF and B2 were cross priming primers, and 5F and 5B were reacted as cross amplification primers and detection probes. For convenient and specific detection by universal nucleic acid detection device, 5F and 5B were labeled with FITC and Biotin, respectively (Fig. 1B). A positive control fragment of bla_{NDM-1} was artificially synthesized based on conservative region of bla_{NDM-1} and cloned in pGEM-T Easy (Promega, Madison, WI). The resulting recombinant plasmid pNDM-1 was used as positive control for reaction optimization and assay development. Amplification conditions were optimized for temperature, concentrations of primers, nucleotides, Mg^{2+}, and betaine. The optimized reaction was carried out in a total volume of 20 μl containing 0.5 μM of PF and B2, 0.3 μM each of primers 5B and 5F, 0.05 μM each of displacement primers F3 and B3, 0.8 mM of dNTP, 1 M betaine (Sigma), 20 mM Tris–HCl(pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1% Triton X-100, 8U Bst DNA polymerase large fragment (New England Biolabs) and positive control or sample templates. The mixture was incubated at 63 °C for 1 h, and then heated at 95 °C for 5 min to stop the reaction. The amplified products were successfully detected by several methods, including electrophoresis on an agarose gel, dispensable universal rapid nucleic acid detection device (Ustar biotechnology, Hangzhou) and double strand DNA dyes. For agarose gel detection, positive amplification resulted in ladder-like bands (Fig. 1C, M. DL2000 DNA Marker, 1. negative, 2. pNDM-1, 3. bla_{NDM-1} positive strain). The amplification products were preferentially detected by a dispensable universal detection device that could prevent contaminations. The device reacted as colloidal gold immunochromatographic assay (GICA). An avidin-labeled DNA probe was used as a ligand that the biotin on the CPA primer could bind to it to give visual colorful bands (Fig. 1D: 1. negative, 2. pNDM-1, 3. bla_{NDM-1} positive strain. C is the strip quality control band, T means testing band). For another visual detection method, SYBR Green I was added to the amplified products

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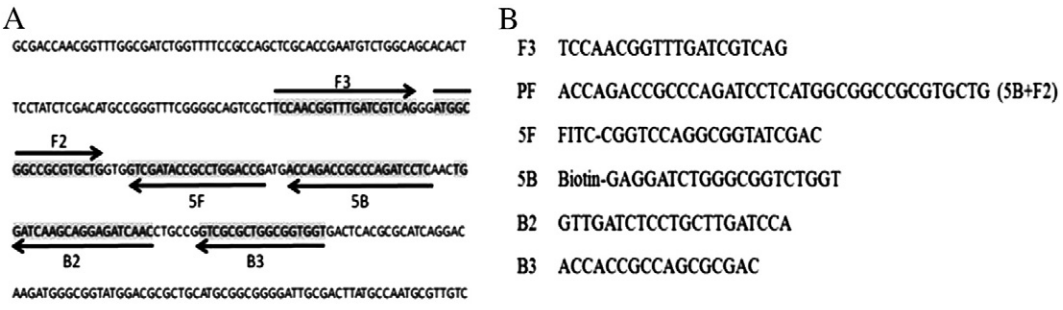

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Sample type	Sample ID	CPA	PCR	DNA Sequencing	Isolates
Stool	ST003	+	-	+	*Enterococcus faecalis*
Stool	ST027	+	+	+	*Acinetobacter schindleri*
Stool	ST118	+	-	+	*Enterococcus faecalis*
Stool	ST216	+	+	+	*Enterococcus faecalis*
Stool	ST247	+	-	+	*Rhodococcus equi*
Stool	ST168	+	-	+	*Rhodococcus equi*
sputum	SP18	+	-	+	*Acinetobacter baumannii*
sputum	SP56	+	-	+	*Acinetobacter baumannii*

Fig. 1. Development and evaluation of the CPA assay for bla_{NDM-1} detection in clinical samples. A: Locations of the six CPA primers in bla_{NDM-1} genes; B: Sequences of CPA primers for bla_{NDM-1}; C: Detection of CPA products by gel electrophoresis; M: DNA marker, 1, negative, 2, pNDM-1, 3, bla_{NDM-1} positive strain; D: Detection of CPA products by dispensable nucleic acid detection device; E: Visual detection of CPA products by adding SYBR green I; and F: Comparison of CPA and PCR for bla_{NDM-1} detection in clinical samples.

and the color changed from purple to green indicated the positive results (Fig. 1E, 1. negative, 2. pNDM-1, 3. bla_{NDM-1} positive strain).

After development of the amplification and detection methods, specificity and sensitivity of CPA were evaluated. Series of 10-fold dilutions of pNDM-1 plasmid DNA with given copies were amplified and detected by CPA. The results showed that the CPA could detect as low as 2 copies of plasmid DNA, being 100 fold more sensitive than PCR. Because GICA was introduced into the detection procedure, the sensitivity of CPA is also higher than that of LAMP(Qi et al., 2012).

The bla_{NDM-1} containing *Acinetobacter baumannii* was cultured and harvested at logarithmic phase. After determination of the cell number, ten-fold serial dilutions (10^0–10^9 CFU/ml) of bacteria were added at 1:1 to bla_{NDM-1} negative sputum, urine, blood and throat swab specimens to generate simulated specimens. DNA were isolated from the simulated specimens and re-suspended in 20 μl distilled water. The detection limit of CPA for the simulated sputum, urine, blood, and throat swab specimens was 100, 50, 100 and 10 CFU/ml respectively, representing about 25, 12.5, 25 and 2.5 CFU per reaction. For simulated stool samples, 1 ml of 10-fold serial dilutions of bacteria was added to 0.5 g of NDM-1 undetectable control stool. Sample DNA were extracted with QIAamp DNA Stool Mini Kit, and NDM-1gene was detected by CPA. The sensitivity of CPA for stool samples was 100 CFU/ml.

In order to evaluate the specificity of the assay, a panel of 25 bacterial strains, including 5 *Escherichia coli*, 5 *Acinetobacter baumannii*, 5 *Klebsiella pneumonia*, 2 *Staphylococcus aureus*, 2 *Stenotrophomonas maltophilia*, 1 *Listeria monocytogenes*, 2 *Salmonella typhimurium*, 2 *Shigella flexneri* and 1 *Yersinia enterocolitica*, were included. These strains were firstly confirmed for absence of bla_{NDM-1} by PCR. Then, template were prepared from these strains and detected by CPA, neither positive nor nonspecific amplification products were detected, indicating that CPA amplification is specific. The CPA was also used to screen for bla_{NDM-1} positive bacteria from a panel of carbapenem resistant *Acinetobacter baumannii* strains. Of the 296 isolates screened, 1 *Acinetobacter baumannii* isolate showed positive result by CPA, the same result as PCR. DNA sequencing of the products confirmed the presence of bla_{NDM-1} in this strain.

A panel of clinical stool and throat swab samples was tested by CPA for the detection of bla_{NDM-1}. A total of 200 stool and 100 sputum samples were tested by both CPA and PCR. Six stool samples and 2 swab samples were shown to be positive by CPA, of which only 2 stool samples were positive by PCR (Fig. 1F). Carbepenem resistant bacteria were isolated from CPA positive samples and screened for bla_{NDM-1} by PCR. Bla_{NDM-1} positive bacteria were successfully isolated from all the CPA positive samples. DNA sequencing of PCR products confirmed the presence of bla_{NDM-1} in the isolated strains. This indicated that CPA showed greatly higher sensitivity and specificity than PCR for bla_{NDM-1} detection in clinical samples. Among the positive samples, 6 strains were isolated from clinical stool samples (3 strains of *Enterococcus*

faecalis, 1 strain of *Acinetobacter schindleri*, 2 strains of *Rhodococcus equi*) and 2 strains were from sputum samples (2 strains of *Acinetobacter baumannii*).

Isothermal amplification techniques are one type of molecular detection methods which are highly sensitive and specific. This type of technique is convenient for on-site and point-of-care detections in clinics. CPA is a recently described isothermal amplification technique. In the present study, we developed a CPA assay for detection of bla_{NDM-1}. The results showed that the assay is highly sensitive and specific for both simulated and clinical samples, and higher than PCR. The high sensitivity and specificity of the CPA assay in detection of bla_{NDM-1} in clinical samples highlighted its potential use in clinical diagnosis, especially for point-of-care diagnosis.

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