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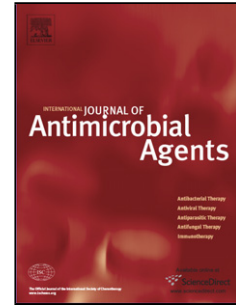
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## Real-time PCR assay allows detection of the New Delhi metallo- $\beta$ -lactamase (NDM-1)-encoding gene in France

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## ABSTRACT

In this study, we report the development of a rapid real-time polymerase chain reaction (PCR) assay with TaqMan<sup>®</sup> probe to detect the New Delhi metallo- $\beta$ -lactamase (NDM-1)-encoding gene directly from bacterial isolates. The specificity of the assay was verified *in silico* as well as with a large panel of 84 clinically relevant bacteria, including the *Klebsiella pneumoniae* NCTC 13443 NDM-1-positive reference strain. Using this assay retrospectively on a local series of 44 *K. pneumoniae* isolates from Marseille Hospitals (France), it was possible to detect and identify an NDM-1-producing *K. pneumoniae* strain isolated from bronchoalveolar lavage in April 2010 from a French patient repatriated from India after a motorbike accident. Standard PCR amplification and sequencing of the entire NDM-1 gene from this isolate was also performed and the amino acid sequence showed 100% homology with the NDM-1 protein from the *K. pneumoniae* reference strain. We believe that this real-time PCR assay would be a powerful tool that could be added to other molecular detection assays such as detection of KPC- or OXA-encoding genes for rapid screening and/or identification of carbapenem-resistant bacterial isolates from patients returning from the Asian continent that could be implemented in a point-of-care strategy.

## 1. Introduction

NDM-1, the New Delhi metallo- $\beta$ -lactamase, is a novel metallo- $\beta$ -lactamase (MBL) conferring resistance to almost all  $\beta$ -lactam antibiotics, including carbapenems, recently identified in *Klebsiella pneumonia* and *Escherichia coli* isolates from a Swedish patient who travelled to New Delhi, India [1]. Following this first description, sporadic cases of infected patients have been reported in India, the UK and the USA [2–4]. Kumarasamy et al. [5] have recently reported the emergence and spread of 180 cases of patients infected with bacteria carrying the NDM-1-encoding gene from Pakistan, India and the UK. Interestingly, most patients from the UK had travelled to India or Pakistan within 1 year and had been hospitalised in these countries, suggesting that these organisms were acquired from a local source in Asia [5]. Since August 2010, other cases have been reported worldwide, including in the USA, Canada, Europe, Japan, Africa, Oman and Australia [6]. The rapid spread and dissemination of these multidrug-resistant (MDR) bacteria worldwide represents a major public health problem, thus the US Centers for Disease Control and Prevention (CDC) has recently planned to add NDM-1-producing MDR bacteria as agents of communicable diseases, and hospitals must immediately report any suspect cases, particularly those for which the patient received medical treatment in India or Pakistan. The aim of this work was to develop a rapid real-time polymerase chain reaction (PCR) assay to detect the NDM-1-encoding gene in bacteria.

## 2. Materials and methods

### 2.1. Bacterial strains and antibiotic susceptibility testing

In total, 128 strains were used in this study, including *K. pneumoniae* reference strain NCTC 13443 carrying the NDM-1 gene, a collection of 83 clinically relevant bacteria previously identified by 16S rRNA gene amplification and sequencing (see below for details) as well as a collection of 44 *K. pneumoniae* clinical isolates from Marseille Hospitals (France). Strains were cultured on trypticase soy agar and one to two colonies were harvested and re-suspended in 500  $\mu$ L of sterile water before DNA extraction and molecular analysis.

Antibiotic susceptibility testing was performed and interpreted according to the Committee for Antimicrobial Testing of the French Society of Microbiology using a VITEK 2 auto system apparatus (bioMérieux, Marcy l'Etoile, France) with Gram-negative susceptibility test cards.

### 2.2. Molecular methods

Specific primers and probe for the real-time PCR assay as well as primers for standard PCR amplification and sequencing of the entire NDM-1 gene are given in Table 1. These primers and TaqMan<sup>®</sup> probe were designed according to NDM-1 gene sequences available in GenBank (*K. pneumoniae* plasmid pKpANDM-1, GenBank accession no. FN396876.1; and *E. coli* megaplasmid blaNDM-1, GenBank accession no. AB571289.1) using Primer3 online software (<http://frodo.wi.mit.edu/primer3>) allowing amplification of a sequence of 155 bp.

Primers used to amplify and sequence the entire NDM-1 gene (KPM<sub>NDM</sub>-F/R) were designed according to Yong et al. [1] in the 4.3-kb region to amplify a 998-bp sequence that overlaps the full NDM-1 gene (Table 1).

Quantification of the NDM-1-encoding gene was performed by quantitative LightCycler<sup>®</sup> real-time PCR (Roche Diagnostics, Meylan, France). MasterMix was prepared according to the manufacturer's instructions. The following LightCycler experimental run protocol was used: denaturation program (95 °C for 15 min); and amplification and quantification program repeated 35 times (95 °C for 30 s, 60 °C for 1 min). PCR was considered negative if the result was  $\geq 35$  threshold cycles (Ct). Sensitivity and reproducibility of the real-time PCR assay were determined using the *K. pneumoniae* reference strain NCTC 13443 carrying the NDM-1 gene.

The specificity of the primers and probe were verified in silico by blastN analysis on the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>). Specificity was also verified in vitro using a local collection of 83 clinically representative bacteria from 13 different genera: *Klebsiella* spp. ( $n = 14$ ); *Enterobacter* spp. ( $n = 4$ ); *Pseudomonas* spp. ( $n = 2$ ); *Acinetobacter* spp. ( $n = 13$ ); *Raoultella* spp. ( $n = 3$ ); *Campylobacter* spp. ( $n = 4$ ); *Citrobacter* spp. ( $n = 12$ ); *Escherichia* spp. ( $n = 7$ ); *Proteus* spp. ( $n = 3$ ); *Providencia* spp. ( $n = 4$ ); *Salmonella* spp. ( $n = 7$ ); *Serratia* spp. ( $n = 7$ ); and *Shigella* spp. ( $n = 3$ ). Among these strains, strains carrying other MBLs include *E. coli* NCTC 13476 carrying the *bla*<sub>IMP</sub> gene, *Pseudomonas aeruginosa* NCTC 13437 carrying the *bla*<sub>VIM-10</sub> and *bla*<sub>VEB-1</sub> genes, *K. pneumoniae* KPC-3 carrying the *bla*<sub>KPC</sub> gene (gift from Prof. Patrice Courvalin, Institut Pasteur, Paris, France) and *Acinetobacter baumannii* strains

carrying *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-24</sub> or *bla*<sub>OXA58</sub>. *Klebsiella pneumoniae* reference strain NCTC 13443 carrying the NDM-1 gene was used as a positive control.

Finally, for standard PCR amplification and sequencing of the entire NDM-1-encoding gene, thermocycler conditions were as follows: an initial denaturation step at 95 °C for 15 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min 30 s. Finally, the tubes were incubated at 72 °C for 5 min to ensure complete synthesis of the entire sequence. Positive PCR products were sequenced using BigDye<sup>®</sup> terminator chemistry on an automated ABI 3730 sequencer (PE Applied Biosystems, Foster City, CA).

### 3. Results

BlastN analysis of the primers and probe designed for development of the real-time PCR assay showed in silico a 100% homology with the NDM-1-encoding gene only. The sensitivity of the real-time PCR using *K. pneumoniae* reference strain NCTC 13443 carrying the NDM-1 gene using serial ten-fold dilutions of a calibrated inoculum at 10<sup>8</sup> colony-forming units/mL was good, with a calibration curve linear from 10–10<sup>8</sup> DNA copies corresponding to 35–18 Ct (Fig. 1). The reproducibility of the quantitative PCR assay was excellent, with a positive PCR at 20.2 ± 0.8 Ct (*n* = 8 different experiments) when testing one to two colonies re-suspended in 500 µL of sterile water. Looking at the in vitro specificity against a panel of 83 clinically relevant bacteria, all real-time PCRs were negative, including strains carrying other MBL-encoding genes. Finally, when tested retrospectively against a collection of 44 *K. pneumoniae* clinical isolates, the real-time PCR assay enabled the detection of a



positive isolate (Ct value = 20.3). To confirm the presence of the NDM-1-encoding gene in this isolate (called KPM\_nasey), the entire gene was amplified and sequenced and showed 100% homology with that of the *K. pneumoniae* NDM-1-encoding gene (GenBank accession no. FN396876.1). The sequence of the NDM-1 gene of our isolate *K. pneumoniae* strain KPM\_nasey has been deposited in GenBank under the accession no. HQ328085. The *K. pneumoniae* KPM\_nasey strain has been also deposited in the CSUR (Collection des Souches de l'Unité des Rickettsies) under reference number CSUR P130.

The *K. pneumoniae* KPM\_nasey strain was in fact isolated in April 2010 in the Intensive Care Unit of Timone Hospital (Marseille, France) from bronchoalveolar lavage of a French patient who had been hospitalised for 2 weeks in March 2010 in New Delhi Hospital (India) after a motorbike accident. He suffered from severe head injury and was colonised by this *K. pneumoniae* strain [6]. Antibiotic susceptibility testing for this strain performed in April 2010 revealed a high resistance phenotype to amoxicillin/clavulanic acid [minimum inhibitory concentration (MIC) > 8 µg/mL], ceftriaxone (MIC > 64 µg/mL), imipenem (MIC = 8 µg/mL), ertapenem (MIC = 256 µg/mL), gentamicin (MIC = 128 µg/mL), amikacin (MIC > 256 µg/mL), pefloxacin (MIC > 32 µg/mL) and sulfamethoxazole/trimethoprim (MIC > 32 µg/mL), but it was susceptible to colistin (MIC = 2 µg/mL) and intermediate to tigecycline (MIC = 1.5 µg/mL).

#### 4. Conclusion

The emergence and rapid dissemination worldwide of carbapenem resistance due to the NDM-1-encoding gene should be seriously monitored in order to avoid any risk of pandemic [6]. In this study we demonstrate the good specificity of a real-time PCR assay for the detection of this alarming gene on a large panel of clinically relevant bacteria that may share carbapenem resistance-encoding genes [7]. Compared with the current existing methods for detection of NDM-1-producers, i.e. screening for carriers of NDM-1-producers with media such as chromID ESBL culture medium and final identification as NDM-1-producers with standard PCR amplification and sequencing [8], the real-time PCR assay has several advantages, including sensitivity, specificity and the possibility to detect NDM-1-producers very quickly (<2 h) either for rapid screening of rectal swabs or for identification of any carbapenem-resistant isolates. We believe that this real-time PCR assay would be an important and powerful tool that could be implemented easily in microbiological laboratories that have molecular facilities, including in point of care, for identification of the NDM-1-encoding gene in any suspect carbapenem-resistant bacterial isolate from patients returning from Asia. Rapid implementation of such molecular assays to current testing strategies should be done in real-time as soon as the sequences of new genes are discovered and tested when a bacterial isolate is atypical or negative for other carbapenemase-encoding genes as demonstrated in this report. As a consequence, the current method should be added to other existing molecular assays targeting other MBLs such as KPC, OXA, VIM and IPM for the identification of carbapenemase-encoding genes.

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**Competing interests**

None declared.

**Ethical approval**

Not required.

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**Fig. 1.** Real-time polymerase chain reaction (PCR) sensitivity test to detect the New Delhi metallo- $\beta$ -lactamase (NDM-1)-encoding gene from *Klebsiella pneumoniae* strain NCTC 13443. CFU, colony-forming units.

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**Table 1**

Primers and probe designed to target the New Delhi metallo- $\beta$ -lactamase (NDM-1)-encoding gene

Primer/probe name	Type of PCR	Sequence	PCR product size (bp)
NDM-1_F	Real-time PCR	3'-GCGCAACACAGCCTGACTTT-5'	155
NDM-1_R		5'-CAGCCACCAAAGCGATGTC-3'	
NDM-1_Probe		6-FAM-CAACCGCGCCCAACTTTGGC-TAMRA	
KPM <sub>NDM-F</sub> <sup>a</sup>	Standard PCR	3'-CATTTGCGGGGTTTTTAATG-5'	998
KPM <sub>NDM-R</sub> <sup>a</sup>		5'-CTGGGTCGAGGTCAGGATAG-3'	

PCR, polymerase chain reaction.

<sup>a</sup> Primers used to amplify and sequence the entire NDM-1 gene (KPM<sub>NDM-F/R</sub>) were designed according to Yong et al. [1] in the 4.3-kb region to amplify a 998-bp sequence that overlaps the full NDM-1 gene.

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Figure 1

