Med Microbiol Immunol (2011) 200:137–141
DOI 10.1007/s00430-011-0189-y

RAPID COMMUNICATION

Real-time PCR assay and a synthetic positive control for the rapid and sensitive detection of the emerging resistance gene New Delhi Metallo-β-lactamase-1 ($bla_{\text{NDM-1}}$)

Alexander Krüttgen · Soheila Razavi · Matthias Imöhl · Klaus Ritter

Received: 6 December 2010 / Published online: 25 February 2011

Abstract Carbapenems are important last-line antibiotics for the treatment of hospital infections. Enterobacteriaceae (such as *Klebsiella pneumoniae* or *Escherichia coli*) expressing the "New Delhi Metallo-β-lactamase" gene $bla_{\text{NDM-1}}$ are resistant to carbapenems and were predicted to become a major global health problem. To cope with this emerging threat, there is a need for rapid and sensitive molecular assays to detect $bla_{\text{NDM-1}}$ in carbapenem-resistant Enterobacteriaceae from clinical isolates. In diagnostic laboratories, real-time PCR is the current gold standard for the sensitive and rapid detection of pathogens. We describe a real-time PCR assay as well as two conventional PCR assays to detect $bla_{\text{NDM-1}}$. Only minute amounts of total DNA extracted from one bacterial colony are sufficient to allow detection of $bla_{\text{NDM-1}}$ by real-time PCR within less than 1 h. We also introduce a chemically synthesized $bla_{\text{NDM-1}}$ gene as a convenient positive control for those laboratories wishing to setup in-house assays for $bla_{\text{NDM-1}}$ detection. Importantly, our study represents a proof of principle for the usefulness of rapidly synthesized genes serving as positive controls for novel diagnostic PCR assays of emerging pathogens during the initial phase after their discovery when biological isolates are still rare and not commonly available.

Keywords PCR · Emerging pathogen · Resistance, synthetic gene, positive control

A. Krüttgen (✉) · S. Razavi · M. Imöhl · K. Ritter
Department of Medical Microbiology,
Medical Faculty, RWTH Aachen University,
Pauwelsstr. 30, 52074 Aachen, Germany
e-mail: akruettgen@ukaachen.de

Introduction

The spread of drug-resistant pathogenic bacteria in hospitals is an important topic in Medical Microbiology and Infectiology [1–4]. In a widely noticed paper, Kumarasamy reported on the emergence of Enterobacteriaceae expressing the so-called New Delhi Metallo-β-lactamase gene, $bla_{\text{NDM-1}}$, conferring resistance to an important class of "last line of defense" antibiotics called carbapenems [5]. Enterobacteriaceae with resistance to carbapenems conferred by $bla_{\text{NDM-1}}$ are likely to spread and are predicted to become a major global health problem [6, 7]. Unfortunately for routine diagnostic laboratories, the initial paper and subsequent case reports did not provide protocols for the PCR-based identification of $bla_{\text{NDM-1}}$ [8–10]. An additional obstacle for establishing an in-house assay for $bla_{\text{NDM-1}}$ in microbiological routine laboratories might currently be the lack of a $bla_{\text{NDM-1}}$ positive control, due to the so far fortunate sparseness of $bla_{\text{NDM-1}}$-positive isolates. Here, we describe three optimized PCR protocols for the sensitive and rapid detection of $bla_{\text{NDM-1}}$ in diagnostic routine laboratories. Moreover, we introduce a convenient positive control, an entirely chemically synthesized $bla_{\text{NDM-1}}$ gene that will be distributed on request. The potential advantages and drawbacks of using synthetic genes as positive controls for diagnostic PCR assays are being discussed.

Materials and methods

A synthetic $bla_{\text{NDM-1}}$ gene as a positive control for PCR assays

The plasmid pMK_blaNDM-1 (3,124 bp) was generated by subcloning the synthetic gene $bla_{\text{NDM-1}}$ (813 bp, Genbank

accession number FN396876.1) synthesized by GeneArt (Regensburg, Germany, www.geneart.com) into the vector pMK-T using a SfiI restriction site. The resulting construct was verified by sequencing and the sequence congruence of the synthesized gene, and the bla_{NDM-1} sequence deposited at Genbank was 100%. This positive control plasmid, pMK_blaNDM-1, contains the promoterless bla_{NDM-1} gene, minimizing potential concerns regarding biosafety risks. This plasmid may be amplified by transformation of commercially available *E. coli* biosafety strains (such as "Top10," Invitrogen) in Luria Broth (LB) or on LB agar plates containing Kanamycin (50 µg/ml) due to the presence of a Kanamycin resistance gene driven by a prokaryotic promoter. DNA from pMK_blaNDM-1-transformed *E. coli* or *Acinetobacter baumannii* was prepared from one bacterial colony picked from an agar plate using a QIAamp mini kit (Qiagen). DNA concentrations were measured using a NanoVue spectrophotometer (GE).

Conventional PCR assays for the detection of bla_{NDM-1}

To establish conventional PCRs for the detection of bla_{NDM-1}, two primer pairs (A and B; Sigma–Aldrich) annealing to the published bla_{NDM-1} sequence were designed (http://frodo.wi.mit.edu/primer3/). BLAST similarity searches were made to exclude non-specific hybridization to target sequences (http://blast.ncbi.nlm.niv.gov). Primer pair A (giving rise to a 206-bp fragment, bp 221–426 of the coding sequence) was Pr1F: 5′-CTTCCAACGG TTTGATCGTC-3′ and Pr2R: 5′-ATTGGCATAAGT CGCAATCC-3′. Primer pair B (giving rise to a 77-bp fragment, bp 154–230 of the coding sequence) was Pr3F: 5′-CCGTTGGAAGCGACTGCCCC-3′ and Pr4R: 5′-CGC CAGCTCGCACCGAATGT-3′. For conventional PCRs, the "HotStarTaq Plus" kit from Quiagen was used. The 25-µl reaction mixture consisted of PCR-grade water, Quiagen-PCR buffer (a final concentration of 2× containing 3 mM $MgCl_2$ was found to be superior compared to 1×), dNTPs (100 µM), primers (0.4 µM), HotStar Taq Plus DNA Polymerase (0.2 Units/µl), and varying amounts of DNA as indicated. Thermal cycling was carried out with a GeneAmp PCR System 9700 Thermocycler (Applied Biosystems). For primer pair A (Pr1F+Pr2R), an initial denaturation (95°C for 5 min) was followed by 30 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 30 s) and a final extension of 1 min at 72°C (the usual 10-min final extension step was found not to improve the sensitivity of the assay). For primer pair B (Pr3F+Pr4R), an initial denaturation (95°C for 5 min) was followed by 30 cycles of a two-step extension (95°C for 30 s, 70°C for 30 s) and a final extension of 1 min at 72°C. The PCR products were analyzed on standard 2% agarose gels containing ethidium bromide.

Real-time PCR assay for bla_{NDM-1}

To increase the sensitivity and rapidity of bla_{NDM-1} detection, we established a real-time PCR assay for the Roche LightCycler. The following primers and Taqman-probe were synthesized by TIB MOLBIOL (Berlin, Germany, http://www.tib-molbiol.com/): NDM-1S: 5′-GCTGGCGGTG GTGACTC-3′; NDM-1L: 5′-GGCAAGCTGGTTCGACA AC-3′; NDM-1TM: 6FAM—TGGCATAAGTCGCAAT CCCCGC—BBQ. The size of the amplicon is 106 bp (342–447 of the coding sequence). The LightCycler FastStart DNA Master HybProbe Kit (Roche) was used to prepare the master mix for each reaction; 1 µl of the 25 mM $MgCl_2$ stock solution (provided with the Roche kit) was added to the final reaction volume of 15 µl; the final concentration of each primer was 0.5 µM; the final concentration of the probe was 0.2 µM. The cycling conditions were initial denaturation for 10 min at 95°C followed by 45 cycles (95°C for 3 s; 55°C for 10 s; 65°C for 60 s).

Results

We first assessed the sensitivity of the PCR assay based on primer pair A by a 10× dilution series of the pMK_blaNDM-1 plasmid with concentrations ranging from 0.4 fg/µl (lane 1) up to 40 pg/µl (lane 6). As shown in Fig. 1a (left-hand side, lane 3), we found that the lower limit of detection of the pMK_blaNDM-1 plasmid was 40 fg/µl (corresponding to 1.2×10^4 plasmid copies per µl of PCR mixture).

Sometimes, inhibitors present in bacterial DNA extracts lower the sensitivity of diagnostic PCR assays. Therefore, we examined whether DNA extracts from Gram-negative bacteria (in this case *A. baumannii,* a typical nosocomial infectious agent) might exert an inhibitory effect on this bla_{NDM-1} PCR assay. As shown in Fig. 1a (right-hand side), this was not the case; in the presence of bacterial DNA extracts, the lower limit of detection remained unchanged (compare lane 3 on the right-hand side and lane 3 on the left-hand side of Fig 1a).

Next, we corroborated that our assay detects the bla_{NDM-1} gene in Enterobacteriaceae. To do so, *E. coli* was transformed with pMK_blaNDM-1 (bla_{NDM-1} *E. coli*), and DNA extracts from a colony on an agar plate was prepared using the QIAamp mini kit. Subsequently, a 10× dilution series of total DNA starting with the lowest concentration of 20 ag/µl (20 attograms/µl = 0.02 femtograms/µl) in lane 1 (corresponding to 2 ag of bla_{NDM-1} *E. coli* DNA per µl of PCR mixture) was subjected to our PCR assay based on primer pair A. As shown in Fig. 1b, the assay was of great specificity as solely one band (corresponding to the 206 bp bla_{NDM-1} fragment) was amplified from bacterial DNA

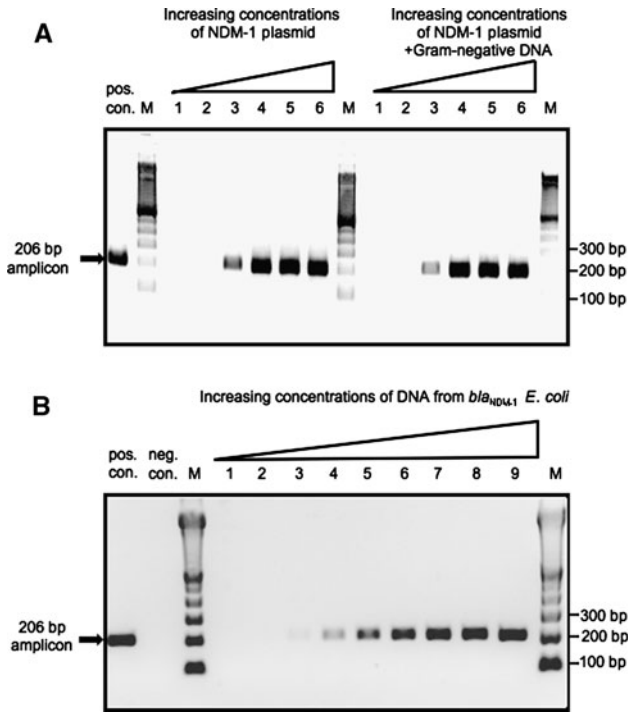

Fig. 1 Detection of $bla_{\text{NDM-1}}$ by conventional PCR with primer pair A. **a** Titration of $bla_{\text{NDM-1}}$ plasmid to reveal the lower limit of sensitivity of PCR; *left-hand side* without *Acinetobacter* genomic extracts, *right-hand side* with *Acinetobacter* genomic extracts (control for potential inhibitory effect of genomic extracts from Gram-negative bacteria on the sensitivity of this $bla_{\text{NDM-1}}$ PCR). *Lane* "*1*": 0.4 fg/µl corresponding to 0.4 fg $bla_{\text{NDM-1}}$ plasmid per µl of PCR mixture; *lane* "*2*": 4 fg/µl; *lane* "*3*": 40 fg/µl; *lane* "*4*": 400 fg/µl; *lane* "*5*": 4 pg/µl; *lane* "*6*": 40 pg/µl. The lower limit of sensitivity using primer pair A was 40 fg/µl $bla_{\text{NDM-1}}$ plasmid (compare appropriate bands in *lane 3*). Comparison of *left-hand side* and *right-hand side* shows no inhibition of amplification by the presence of bacterial DNA. *M* Marker (100-bp ladder), *pos. con*. positive control, *neg. con*. negative control. **b** Detection of the $bla_{\text{NDM-1}}$ gene in $bla_{\text{NDM-1}}$-transformed *E. coli*; titration to reveal the lower limit of sensitivity for $bla_{\text{NDM-1}}$ detection in DNA extracts from $bla_{\text{NDM-1}}$-transformed *E. coli*. *Lane* "*1*": 20 ag/µl corresponding to 20 ag of total *E. coli* DNA per µl of PCR reaction; *lane* "2": 0.2 fg/µl; *lane* "*3*": 2 fg/µl; *lane* "*4*": 20 fg/µl; *lane* "*5*": 0.2 pg/µl; *lane* "*6*": 2 pg/µl; *lane* "*7*": 20 pg/µl; *lane 8*: 0.2 ng/µl; *lane 9*: 2 ng/µl. The lower limit of sensitivity using primer pair A was 2 fg/µl of *E. coli* DNA extracts (see weak band in *lane 3*). *M* Marker, *pos. con.* positive control, *neg. con.* negative control

extracts. Moreover, we also demonstrated a high sensitivity as $bla_{\text{NDM-1}}$ was detectable in as little as 2 fg/µl of PCR mixture (lane 3), corresponding to 50-fg total DNA in a reaction volume of 25 µl. As the QIAamp mini kit normally yields 10 ng/µl (=10.000.000 fg/µl) of total bacterial DNA in an elution volume of 200 µl, the sensitivity of the PCR assay is overwhelmingly sufficient for our purpose. Our assay is also of great specificity as no band was amplified in DNA prepared from non-transformed control *E. coli* (data not shown) or *A. baumannii* (see Fig. 1a, right-hand side, lane 1 and lane 2).

In theory, a single primer pair might give false-negative PCR results due to mutations in bacterial target sequences. Therefore, we wished to establish a second protocol based on a different set of primers. Next, we tested the sensitivity of a PCR assay based on a second primer pair (coined primer pair "B" characterized by a high annealing temperature of 70°C allowing rapid two-step PCR) in a similar way as described earlier for primer pair A. As shown in Fig. 2a (left-hand side, lane 2), we found the lower limit of detection of the plasmid to be 4 fg/µl (which corresponds to 1.2×10^3 plasmid copies per µl of PCR mixture), appearing to be a bit more sensitive than primer pair A. Subsequently, we examined whether DNA extracts from Gram-negative bacteria (as exemplified by *Acinetobacter baumannii*) might exert an inhibitory effect on this $bla_{\text{NDM-1}}$ PCR assay. As shown in Fig. 2a, (right-hand side), this was not the case (compare lane 2 on the right-hand side with lane 2 on the left-hand side). Next, we subjected DNA extracted from the $bla_{\text{NDM-1}}$ *E. coli* strain to this PCR assay, in a similar way as described earlier for primer pair A. As shown in Fig. 2b, also this PCR assay was of great specificity as solely one band (corresponding to the 77-bp $bla_{\text{NDM-1}}$ fragment) was amplified from these bacterial DNA extracts. Moreover, we also demonstrated a high sensitivity of this assay, because $bla_{\text{NDM-1}}$ was detectable in as little as 2 fg/µl of total $bla_{\text{NDM-1}}$ *E. coli* DNA extracts (lane 3). As discussed earlier, this is a minute fraction of the bacterial DNA obtained from one bacterial colony picked from an agar plate and extracted with the QIAamp mini kit. Moreover, this PCR assay is specific as no band was discovered in total DNA extracts prepared from non-transformed control *E. coli* (data not shown) or *A. baumannii* (see lane 1 in Fig. 2a right-hand side).

Real-time PCR represents the current gold standard in molecular diagnostics. To further increase the sensitivity and rapidity of $bla_{\text{NDM-1}}$ detection, we established a real-time PCR assay for the Roche LightCycler. We found the limit of sensitivity to be at least 15 $bla_{\text{NDM-1}}$ copies per microliter of reaction mixture (data not shown). Next, we subjected our DNA extracts from *E. coli* harboring $bla_{\text{NDM-1}}$ to this assay: $bla_{\text{NDM-1}}$ was readily detectable when 2.5 ng of total bacterial DNA was present in a 15-µl real-time PCR mixture(final concentration 170 pg total DNA/µl of reaction), whereas no fluorescence signal appeared in DNA extracts from non-transformed control *E. coli* (data not shown). Moreover, we also confirmed this finding with DNA extracted from a naturally occurring *Klebsiella pneumoniae* isolate harboring $bla_{\text{NDM-1}}$ (kindly provided by the National Reference Laboratory for multidrug-resistant Gram-negative bacteria, Bochum, Germany; data not shown). Thus, our real-time PCR offers the necessary sensitivity and specificity for rapid identification of $bla_{\text{NDM-1}}$in total DNA extracted from a bacterial colony.

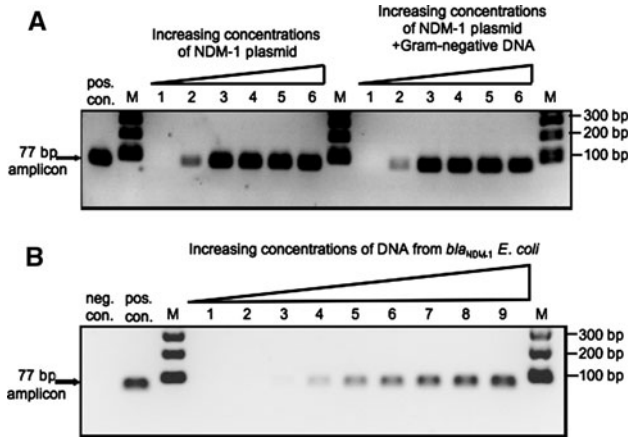

Fig. 2 Detection of $bla_{\text{NDM-1}}$ by conventional PCR with primer pair B. **a** Titration of $bla_{\text{NDM-1}}$ plasmid to reveal the lower limit of sensitivity of PCR; *left-hand side* without *Acinetobacter* genomic extracts, *right-hand side* with *Acinetobacter* genomic extracts (control for potential inhibitory effect of genomic extracts from Gram-negative bacteria on the sensitivity of this $bla_{\text{NDM-1}}$ PCR). *Lane* "*1*": 0.4 fg/µl corresponding to 0.4 fg $bla_{\text{NDM-1}}$ plasmid per µl of PCR mixture; *lane* "*2*": 4 fg/µl; *lane* "*3*": 40 fg/µl; *lane* "*4*": 400 fg/µl; *lane* "*5*": 4 pg/µl; *lanes* "*6*": 40 pg/µl. The lower limit of sensitivity using primer pair B was 4 fg $bla_{\text{NDM-1}}$ plasmid per µl of PCR mixture (compare the appropriate bands in *lane 2*). Comparison of *left-hand side* and *right-hand side* shows no inhibition of amplification by the presence of bacterial DNA. *M* Marker (100 bp ladder), *pos. con.* positive control, *neg. con.* negative control. **b** Detection of the $bla_{\text{NDM-1}}$ gene in $bla_{\text{NDM-1}}$-transformed *E. coli*; titration to reveal the lower limit of sensitivity for NDM-1 detection in DNA extracts from $bla_{\text{NDM-1}}$-transformed *E. coli. Lane* "*1*": 20 ag/µl corresponding to 20 ag of total *E. coli* DNA per µl of PCR mixture; *lane* "*2*": 0.2 fg/µl; *lane* "*3*": 2 fg/µl; *lane* "*4*": 20 fg/µl; *lane* "*5*": 0.2 pg/µl; *lane* "*6*": 2 pg/µl; *lane* "*7*": 20 pg/µl; *lane 8*: 0.2 ng/µl; *lane 9*: 2 ng/µl. The lower limit of sensitivity using primer pair A was 2 fg/µl of total *E. coli* DNA harboring $bla_{\text{NDM-1}}$ (see weak band in *lane 3*). *M* Marker, *pos. con*. positive control, *neg. con*. negative control

Discussion

Enterobacteriaceae-expressing carbapenemases are a major threat to the global health system as this class of antibiotics is of major importance as a last-line therapy of nosocomial infections. The recent discovery of a novel carbapenemase called New Delhi Metallo-β-lactamase, NDM-1, was widely noticed and even reported by popular media. Although the number of reported cases is very low, diagnostic laboratories need to prepare themselves for future nosocomial outbreaks.

The recently published guidelines for the detection of carbapenemases in Enterobacteriaceae include an initial screening step followed by a PCR-based test to detect carbapenemase genes [11]. Although it is straightforward to identify carbapenem-resistant bacteria by conventional susceptibility testing, there is an acute need for rapid and sensitive molecular assays to detect the novel $bla_{\text{NDM-1}}$ gene in carbapenem-resistant Enterobacteriaceae from clinical isolates. To close this gap, we describe a sensitive and rapid real-time PCR assay as well as two conventional PCR assays to detect $bla_{\text{NDM-1}}$. Minute amounts of total DNA extracted from one bacterial colony picked from an agar plate are sufficient to detect $bla_{\text{NDM-1}}$ by LightCycler PCR in less than 1 h or by conventional PCR in 3 h. The combination of 3 different PCR primer pairs and two different PCR techniques should minimize the risk of false-negative or false-positive PCR results. As these assays allow for the ultrasensitive detection of $bla_{\text{NDM-1}}$ in bacterial colonies picked from agar plates, future studies should explore if these fast assays might also allow direct detection (without previous culture) of $bla_{\text{NDM-1}}$ in clinical samples such as swaps, stool, or blood. In a similar way, some hospitals are currently performing real-time PCR assays to screen incoming patients for methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *Enterococcus* (VRE).

Recent technical breakthroughs brought about that direct synthesis of genes (www.dna2.0.com; www.geneart.com) is rapidly becoming the most efficient and cheapest way to make functional genetic constructs [12]. For three reasons, this novel technical development appears to be of great relevance to microbiological laboratories:

(i) It allows for the rapid synthesis of genes serving as positive controls for novel diagnostic PCR assays of emerging pathogens. This seems especially important during the initial phase after discovery of a novel biological threat, at a time when biological isolates are still rare and not commonly available. The present study provides a proof of principle for this scenario. In our laboratory, no $bla_{\text{NDM-1}}$-expressing bacterial strain was initially available. We bypassed this problem by using a synthetic $bla_{\text{NDM-1}}$ gene (transformed into *E. coli*) as a "surrogate positive control" to setup and subsequently optimize our novel PCR assays.

(ii) The use of synthetic genes as positive controls seems also highly appropriate when dealing with well known but very hazardous organisms. As an impressive example, Carrera and Sagripanti recently reported the generation of a plasmid encoding specific synthetic DNA fragments of *Yersinia pestis*, *Francisella tularensis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Rickettsia sp.*, *Coxiella burnetii*, *Brucella sp.*, *Bacillus anthracis*, and *Variola* virus, allowing this single plasmid to be used as a versatile multifunctional positive control for the PCR-based amplification of these dangerous microorganism, without actually requiring access to these agents [13, 14].

(iii) The use of synthetic genes could catalyze scientific progress in those research areas where concerns about scientific competition obstruct the distribution of useful research reagents such as plasmids or strains.

Despite these advantages, the use of synthetic genes as positive controls for the detection of carbapenemases may also bring about two points to be kept in mind:

(i) Although the use of a synthetic gene as positive control reaction demonstrates that the PCR assay works and all reagents are functional, a carbapenemase-positive specimen might still give a false-negative result because PCR-inhibitory agents could potentially co-purify along together with bacterial DNA during the extraction procedure. To control for this possibility, an additional control PCR reaction should always be included (compare Figs. 1, 2) in which a defined amount of the synthetic gene is added to the bacterial DNA extract: if the bacterial DNA extracts contained PCR inhibitors, this will become apparent because the amplification of the added synthetic gene will be impaired.

(ii) Although a rapidly available synthesized gene is warmly welcomed as a "substitute positive control" during the initial phase after discovery of a novel biological threat when biological isolates are still rare and not commonly available, a subsequent validation of the novel PCR protocol using a "real" strain isolated from patients appears highly recommended. To this end, after initially setting up our novel PCR assays using the synthetic $bla_{\text{NDM-1}}$ gene and a $bla_{\text{NDM-1}}$-transformed *E.coli*, we later on validated our PCR assays using a $bla_{\text{NDM-1}}$-expressing *Klebsiella pneumoniae* strain provided by a reference laboratory.

In summary, we provide protocols for $bla_{\text{NDM-1}}$ PCR assays and introduced a chemically synthesized $bla_{\text{NDM-1}}$ gene (www.geneart.com, Regensburg, Germany) as a convenient positive control for those laboratories wishing to establish molecular assays for the detection of $bla_{\text{NDM-1}}$. This reagent will be distributed and should—along with our protocols—facilitate the molecular identification of NDM-1-expressing Enterobacteriaceae in routine diagnostic laboratories. Importantly, our study presents the proof of principle for the usefulness of rapidly synthesized genes serving as positive controls for novel diagnostic PCR assays of emerging pathogens during the initial phase after their discovery when biological isolates are still rare and not commonly available.

Acknowledgments We wish to thank Dr. M. Kaase (National Reference Laboratory for multidrug-resistant Gram-negative bacteria, Bochum, Germany; director: Prof. Dr. S. Gatermann) for kindly providing a $bla_{\text{NDM-1}}$ *Klebsiella pneumoniae* isolate.

References

1. Higgins PG, Dammhayn C, Hackel M, Seifert H (2010) Global spread of carbapenem-resistant *Acinetobacter baumannii*. J Antimicrob Chemother 65:233–238
2. Bruhn C (2010) NDM-1-producing bacteria–danger due to "super germs"? Dtsch Med Wochenschr 135:38
3. Leverstein-Van Hall MA, Stuart JC, Voets GM, Versteeg D, Tersmette T, Fluit AC (2010) Global spread of New Delhi metallo-beta-lactamase 1. Lancet Infect Dis 10:830–831
4. Webster PC (2010) Global action urged in response to new breed of drug-resistant bacteria. Cmaj 182:1602–1603
5. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis 10:597–602
6. Bush K (2010) Alarming beta-lactamase-mediated resistance in multidrug-resistant Enterobacteriaceae. Curr Opin Microbiol 13:558–564
7. Rolain JM, Parola P, Cornaglia G (2010) New Delhi metallo-beta-lactamase (NDM-1): towards a new pandemia? Clin Microbiol Infect 12:1699–1701
8. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR (2009) Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob Agents Chemother 53:5046–5054
9. Karthikeyan K, Thirunarayan MA, Krishnan P (2010) Coexistence of blaOXA-23 with blaNDM-1 and armA in clinical isolates of *Acinetobacter baumannii* from India. J Antimicrob Chemother 65:2253–2254
10. Deshpande P, Rodrigues C, Shetty A, Kapadia F, Hedge A, Soman R (2010) New Delhi Metallo-beta lactamase (NDM-1) in Enterobacteriaceae: treatment options with carbapenems compromised. J Assoc Physicians India 58:147–149
11. Cohen Stuart J, Leverstein-Van Hall MA (2010) Guideline for phenotypic screening and confirmation of carbapenemases in Enterobacteriaceae. Int J Antimicrob Agents 36:205–210
12. Villalobos A, Ness JE, Gustafsson C, Minshull J, Govindarajan S (2006) Gene Designer: a synthetic biology tool for constructing artificial DNA segments. BMC Bioinformatics 7:285
13. Carrera M, Sagripanti JL (2009) Artificial plasmid engineered to simulate multiple biological threat agents. Appl Microbiol Biotechnol 81:1129–1139
14. Carrera M, Sagripanti JL (2009) Non-infectious plasmid engineered to simulate multiple viral threat agents. J Virol Methods 159:29–33