Characterization of the first isolate of *Klebsiella pneumoniae* carrying New Delhi metallo-\(\beta\)-lactamase and other extended spectrum \(\beta\)-lactamase genes from Malaysia

The antibiotic resistance mechanisms in Gram-negative bacteria, particularly the *Enterobacteriaceae*, are mainly due to the production of \(\beta\)-lactamases, which render almost all \(\beta\)-lactams ineffective. Carbenapen-resistant *Enterobacteriaceae* (CRE) producing metallo-\(\beta\)-lactamases have been reported in many countries and they are also emerging in the Asia Pacific region (Fukigai et al., 2007; Koh et al., 2001).

A new metallo-\(\beta\)-lactamase gene, *bla*\text{NDM-1}, was first described in a *Klebsiella pneumoniae* isolate from a Swedish patient who had been to India for medical treatment (Yong et al., 2009). A large study on multidrug-resistant *Enterobacteriaceae* isolates from India, Pakistan and the UK showed that strains harboring the NDM-1 gene are not an uncommon occurrence in these countries (Kumarasamy et al., 2010). Since then, many reports have been published on NDM-1-positive CRE in regions such as Australia, Japan and Taiwan (Poirel et al., 2010; Chihara et al., 2011; Wu et al., 2010).

The Malaysian National Surveillance of Antibiotic Resistance surveillance data in 2011 showed that the frequency of carbapenem resistance in *K. pneumoniae* isolates was less than 0.3% (Ministry of Health Malaysia, 2011). The appearance of NDM-1 in a *K. pneumoniae* strain isolated from a patient in one of our hospitals was indeed very worrying as it has the potential to spread.

We have described here the phenotypic and molecular characteristics of our first isolate of *K. pneumoniae* carrying the NDM-1 gene.

*K. pneumoniae* strain VF408/10 was isolated from a urine sample from a 24-year-old female who has underlying acute myeloid leukaemia. Her urine culture collected under aseptic conditions showed pure growth of *K. pneumoniae*. The strain was susceptible to imipenem but was resistant to meropenem and ertapenem. It was sent for verification of antibiotic resistance to the Bacteriology Unit, Institute for Medical Research, Kuala Lumpur.

At the Bacteriology Unit, antibiotic disc susceptibility tests were carried out against amoxicillin/clavulanic acid, ampicillin, ampicillin/sulbactam, cefazidime, cefoperazone, cefoperazone/sulbactam, cefotaxime, cefuroxime, ceftipime, gentamicin, amikacin, netilmicin, piperacillin/tazobactam, nitrofurantoin, ciprofloxacin, trimethoprim/sulfamethoxazole, imipenem, meropenem and ertapenem following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011). MICs of imipenem, meropenem, colistin and polymixin B were determined by the E-test (AB Biodisk). An MIC of \(\geq 4\) \(\mu\)g ml\(^{-1}\) indicates resistance to both carbapenems. For colistin and polymixin B, an MIC \(\leq 2\) \(\mu\)g ml\(^{-1}\) indicates susceptibility.

Modified-Hodge test (MHT) was carried out as described by the CLSI (2011). EDTA-disc synergy tests were carried out as described by Lee et al. (2001). A suspension (0.5 McFarland standard) of the test organism was spread onto Mueller–Hinton agar and 10 \(\mu\)g imipenem was placed 15 mm away from a 1.5 mg EDTA disc on the agar surface. The presence of enhanced inhibition zones between the discs after an overnight incubation was interpreted as a positive test. For the imipenem/ imipenem + EDTA disc test methods, discs containing 10 \(\mu\)g imipenem with and without the addition of 0.5 M EDTA were used. Increase in inhibition zone diameter from 8 to 26 mm produced in the presence of EDTA was considered positive (Petropoulou et al., 2006).

Screening for the NDM-1 gene was carried out with primer pairs NDM1_AF

\[5’-CGTCCATACCGCCATCTTTGTC-3’\]

and NDM1_AR5

\[3’-CTAGGAATTGCCC-AATATTATGCACCC-5’\]. These primer pairs targeted the last 391 bp of the NDM-1 gene. A second primer pair NDM1_BF

\[5’-TCAGGGCAGCTTG-TGGC-3’\] and NDM1_BR

\[5’-CGCCAGATCTCAACTGGA-3’\], which amplified the first 520 bp of the NDM-1 gene, was designed in order to obtain the entire NDM-1 gene sequence. Both primer sets were designed from GenBank nucleotide accession number FN936876. The PCR was carried out in 25 \(\mu\)l reaction buffer, containing DNA (1.0 \(\mu\))l, primers (2 mM each), dNTPs (0.8 mM), Taq DNA polymerase (2.5 U Ampli Taq Gold; Roche Diagnostics), MgCl\(_2\) (3 mM) and DMSO (10%). The cycling conditions included 5 min denaturation at 94 °C (1 cycle); 30 s denaturation at 94 °C, 30 s annealing at 56 °C and 1 min polymerization at 72 °C (35 cycles); followed by a 10 min extension at 72 °C. The presence of the *bla*\text{NDM-1}* gene was determined following the method described by Yigit et al. (2002). The *bla*\text{SHV}, *bla*\text{TEM} and *bla*\text{CMY} genes were detected using the primers as described in Bouallégue-Godet et al. (2005). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and were sent for DNA sequencing. The nucleotide sequences were analysed with MEGA software, version 4.0 (Tamura et al., 2007). The BLAST program of the National Center for Biotechnology Information was used for sequence alignment and database searches.

Seven housekeeping genes, *rpoB*, *gapA*, *mdh*, *pgi*, *phaE*, *infB* and *tonB*, were amplified to determine the clonality of the strain using specific multilocus sequence typing (MLST) primers, as described by the Institut Pasteur (2011).

Antibiotic disc susceptibility tests showed resistance to all the tested carbapenems, cephalosporins, piperacillin/tazobactam,
trimethoprim/sulfamethoxazole and aminoglycosides [except amikacin (intermediate)], and sensitivity to nitrofurantoin and ciprofloxacin. The strain was also sensitive to colistin and polymyxin B, with MICs of 0.094 and 13.0 μg ml⁻¹, respectively. The MICs of imipenem and meropenem were 12 and >32 μg ml⁻¹, respectively. The MHT, EDTA-disc synergy test and imipenem/ imipenem + EDTA disc test methods were all positive for this strain. The sequences of the PCR products from isolate V408/10 were 100% identical to the respective segments of NDM-1 (GenBank accession no. FN396876). This strain was also positive for other β-lactamase genes, namely CTX-M 15, TEM-1 and SHV-11. No PCR product was observed with KPC and CMY primers. The full NDM-1 gene sequence of VF408/10 K. pneumoniae has been submitted to GenBank with the accession no. HQ738352. MLST showed that the strain belonged to clone ST17.

The strain initially tested sensitive to imipenem using the disc susceptibility test. Imipenem disc susceptibility testing is not a reliable indicator for carbapenemase producers. It has been shown to be a poor indicator for K. pneumoniae carbapenemase (KPC)-mediated resistance compared with tests for meropenem and ertapenem (CLSI, 2011; Anderson et al., 2007). Some automated susceptibility testing systems were also unable to give accurate results for KPC-producing K. pneumoniae (Tenover et al., 2006). The same experience was obtained in testing VIM-1-producing K. pneumoniae isolates (Giakkoupi et al., 2005). In this case, the imipenem disc susceptibility test which was carried out manually at the hospital’s microbiology laboratory was initially reported as sensitive. Antibiotic susceptibility testing by disc and E-test methods carried out in our laboratory showed that the strain was resistant to imipenem, which means that discrepancies in sensitivity testing can occur for NDM-1-producing K. pneumoniae.

The MHT, which is the confirmatory phenotypic test for carbapenemase producers, is positive for both KPC- and NDM-1-producing strains. However, it can be differentiated by the use of EDTA, a known inhibitor of metallo-β-lactamase, which chelates the zinc component of the enzyme.

The presence of β-lactamase genes CTX-M-15 and TEM-1 has been described in NDM-1-positive Escherichia coli strains from Australia and Hong Kong, respectively (Poirel et al., 2010; Ho et al., 2011). β-Lactamase genes (bla) are normally carried on plasmids and transposons, and can be easily transferred. Further characterization needs to be carried out to determine whether NDM-1 and other bla genes are carried on the same plasmid.

The K. pneumoniae isolate was typed as ST17, which is different from the first reported NDM-1-carrying K. pneumoniae isolate, which was ST14. The ST17 clone, to the best of our knowledge, has not been reported to harbour the NDM-1 gene but is known to harbour other carbapenemase genes such as VIM-1 and KPC (Daoud et al., 2008; Giakkoupi et al., 2011).

The patient was previously admitted into a hospital located south of Peninsular Malaysia, where she was diagnosed with acute myeloid leukaemia. She defaulted on her follow-up after the first course of chemotherapy, and was readmitted into this haematology specialist hospital for severe anaemia and low-grade fever. She could have acquired the K. pneumoniae NDM-1-positive strain from exposure to the hospital environment. Blood and urine cultures and sensitivity testing were routinely conducted on all febrile haematology oncology patients. The blood culture was negative and she did not have any symptoms of urinary tract infection. Her repeat urine culture taken 3 days later was negative and she was assumed to be colonized by bacteria and no treatment was given. A rectal swab culture for screening of K. pneumoniae NDM-1 carriage was also negative. Neither she nor her close relatives had a history of travelling outside Malaysia. Previous reports have linked patients with a history of travelling or seeking treatment in India and Pakistan (Yong et al., 2009; Kumarasamy et al., 2010; Poirel et al., 2010; Cohen Stuart et al., 2010). However, not all patients infected with NDM-1-positive bacteria have a history of hospital admission in India. NDM-1 β-lactamase-producing bacteria have also been isolated from water and sewage seepage in India (Walsh et al., 2011); a finding that shows that the gene can persist in the environment and is a potential source for dissemination. An asymptomatic patient carrying CRE may transmit the strain to other patients, and therefore it is important to place the patient under isolation. The decision to discontinue isolation precautions can be made based on negative surveillance culture but it is often difficult because CRE can be carried persistently in the gut and the duration of colonization can vary from a few days to many months.

With the discovery of what we believe to be the first isolate of NDM-1-positive K. pneumoniae in Malaysia, the Ministry of Health Malaysia has developed a new guideline for the detection and management of CRE to be adhered to by all hospitals. Prudent surveillance is currently carried out to prevent the establishment of NDM-1-carrying bacteria in our hospital environments.

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