Identification of New Delhi Metallo-β-lactamase 1 in Acinetobacter lwoffii of Food Animal Origin

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Identification of New Delhi Metallo-β-lactamase 1 in Acinetobacter Iwofii of Food Animal Origin

Abstract

Background

To investigate the presence of metallo-β-lactamase (MBL) genes and the genetic environment of the New Delhi metallo-β-lactamase gene blaNDM-1 in bacteria of food animal origin.

Methodology/Principal Findings

Gram-negative bacteria with low susceptibility to imipenem (MIC > 8 µg/mL) were isolated from swab samples collected from 15 animal farms and one slaughterhouse in eastern China. These bacteria were selected for phenotypic and molecular detection of known MBL genes and antimicrobial susceptibility testing. For the blaNDM-1 positive isolate, conjugation and transformation experiments were carried out to assess plasmid transfer. Southern blotting was conducted to localize the blaNDM-1 genes, and DNA sequencing was performed to determine the sequences of blaNDM-1 and the flanking genes. In total, nine Gram-negative bacteria of four different species presented a MBL phenotype. blaNDM-1 was identified on a mobile plasmid named pAL-01 in an Acinetobacter Iwofii isolate of chicken origin. Transfer of pAL-01 from this isolate to E. coli J53 and JM109 resulted in resistance to multiple β-lactams. Sequence analysis revealed that the blaNDM-1 gene is attached to an intact insertion element ISAba125, whose right inverted repeat (IR-R) overlaps with the promoter sequence of blaNDM-1. Thus, insertion of ISAba125 likely enhances the expression of blaNDM-1.

Conclusion

The identification of a blaNDM-1-carrying strain of A. Iwofii in chickens suggests the potential for zoonotic transmission of blaNDM-1 and has important implications for food safety.

Keywords

Gram negative bacteria, Acinetobacter, Antibiotics, Chickens, Polymerase chain reaction, Antibiotic resistance, Southern blot, DNA sequence analysis

Disciplines

Animal Diseases | Bacteriology | Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments

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Identification of New Delhi Metallo-\(\beta\)-lactamase 1 in *Acinetobacter lwoffii* of Food Animal Origin

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Abstract

**Background:** To investigate the presence of metallo-\(\beta\)-lactamase (MBL) genes and the genetic environment of the New Delhi metallo-\(\beta\)-lactamase gene *bla*\(_{NDM-1}\) in bacteria of food animal origin.

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**Conclusion:** The identification of a *bla*\(_{NDM-1}\)-carrying strain of *A. lwoffii* in chickens suggests the potential for zoonotic transmission of *bla*\(_{NDM-1}\) and has important implications for food safety.

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Conclusion: The identification of a *bla*\(_{NDM-1}\)-carrying strain of *A. lwoffii* in chickens suggests the potential for zoonotic transmission of *bla*\(_{NDM-1}\) and has important implications for food safety.

Introduction

Metallo-\(\beta\)-lactamases (MBLs) in clinical Gram-negative organisms are an important threat to public health. MBLs, which require divalent cations (usually zinc ions) as metal cofactors for enzymatic activity, can hydrolyze all \(\beta\)-lactams including carbapenems (except aztreonam) [1]. In 2009, a novel MBL enzyme, named New Delhi metallo-\(\beta\)-lactamase (NDM-1, encoded by *bla*\(_{NDM-1}\)), was identified in plasmids from *Klebsiella pneumoniae* and *Escherichia coli* isolates recovered from a Swedish patient previously hospitalized in India [2]. Recently, *bla*\(_{NDM-1}\)-harboring strains of *Enterobacteriaceae* have been identified worldwide, with most reports indicating that the isolates originated from the Indian subcontinent with hospital or community acquisition [3] or from the Balkan countries [4]. However, NDM-1 positive strains have also been reported in patients with no known contact with these areas [5]. Coexistence of *bla*\(_{NDM-1}\) with the class D carbapenemase gene *bla*\(_{OXA-23}\) in clinical isolates of *Acinetobacter baumannii* was first detected in India [6]. Subsequently, five *bla*\(_{NDM-1}\)-harboring *A. baumannii* clinical strains were reported in five different provinces in China [7,8]. A multidrug resistant *Acinetobacter lwoffii* strain carrying a *bla*\(_{NDM-1}\) plasmid was also isolated from the urine of a female patient in China [9]. Although China borders India and Pakistan, there was no evidence that the infected patients had any contact with the Indian subcontinent.

Recently, *bla*\(_{NDM-1}\) was found in a number of bacterial species isolated from water and seepage samples in central New Delhi, suggesting it is widely disseminated in the local environment [10]. In addition, *bla*\(_{NDM-1}\)-carrying bacteria have also been reported as gut colonizers in patients with no clinical symptoms, suggesting the possible transmission of *bla*\(_{NDM-1}\) via the fecal-oral route, and that fecal flora may serve as a reservoir for *bla*\(_{NDM-1}\) dissemination [3,10,11]. These findings suggest that the spread of the NDM-1 resistance determinant is much wider than previously realized. However, no information is available on the distribution of NDM-1 in food producing animals. Here we investigated the prevalence of Gram-negative bacteria with low susceptibility to imipenem in...
food producing animals, and determined whether MBL genes, including blaNDM-1, were present in these animal isolates.

**Results**

Prevalence of Gram-negative bacteria with low susceptibility to imipenem in food producing animals

In total, nine isolates with very low susceptibility to imipenem (MIC > 8 µg/mL for imipenem), obtained from eight of the 396 samples, were confirmed as Gram-negative bacteria. The isolates were identified by ATB 32 GN, ATB 32E and 16S rDNA sequencing analysis as S. maltophilia (n = 3), Chryseobacterium indolgenes (n = 3), Myroides odoratimimus (n = 1), Myroides odoratus (n = 1), and A. lwoffii (n = 1). Eight of these isolates came from pig samples, while the A. lwoffii isolate was derived from a chicken sample. No imipenem-resistant Enterobacteriaceae strains were identified in this study. Two isolates, M. odoratimimus LYP-BCYg6a and M. odoratus LYP-BCYg6b, were collected from one rectal swab sample from a pig farm.

Characterization of the nine Gram-negative bacteria with low susceptibility to imipenem

All nine Gram-negative isolates presented high MIC values for three tested carbapenems, including imipenem (ranging from 32–128 µg/mL), meropenem (16–64 µg/mL), and ertapenem (16–512 µg/mL). Imipenem-EDTA double-disc synergy tests and E-test MBL strip tests confirmed that all nine isolates were MBL-positive. However, PCR analysis using primers specific for known mobile and chromosomal MBL genes revealed that only the A. lwoffii strain SGC-HZ9, of chicken origin, was blaNDM-1 positive. This blaNDM-1-harboring strain was resistant not only to all tested β-lactams (except aztreonam, 8 µg/mL) but also to ciprofloxacin (8 µg/mL), tetracycline (32 µg/mL), kanamycin (64 µg/mL), and chloramphenicol (32 µg/mL), while remaining susceptible to gentamicin (0.5 µg/mL) and polymyxin B (0.016 µg/mL) (Table 1). In addition, two ubiquitous free-living bacteria (one Myroides odoratimimus LYP-BCYg6a and one Myroides odoratus isolate LYP-BCYg6b) harbored a chromosomal MBL gene, blaoM-1. Six isolates with a MBL phenotype did not contain any of the tested MBL genes.

Phenotypic and plasmid characterization of A. lwoffii SGC-HZ9

Plasmids of A. lwoffii SGC-HZ9 were extracted and separated by pulsed field gel electrophoresis (PFGE). Subsequent Southern blotting analysis of the PFGE gel revealed hybridization of a blaNDM-1-specific probe to a ~270 kb plasmid, which was designated pAL-01 (Figure 1). Plasmid pAL-01 was successfully transferred from SGC-HZ9 to E. coli J53 using filter mating and to E. coli JM109 using electroporation. The size of the plasmid recovered from transconjugant JM109-SGC-HZ9 was consistent with that observed using PFGE, while two plasmid bands were identified in transconjugants J53-SGC-HZ9 (approximately 260 kb and 270 kb) (Figure 1A). Southern blotting using the blaNDM-1 probe further confirmed the presence of pAL-01 in transconjugant JM109-SGC-HZ9, while in transconjugant J53-SGC-HZ9, the probe hybridized to a 260 kb plasmid, suggesting unknown rearrangements had been occurred in the transconjugants with respect to pAL-01 (Figure 1B). Susceptibility tests revealed that both the transconjugant and transformant presented resistance or decreased susceptibility to all tested β-lactams (except aztreonam) as compared with the recipients E. coli J53 and JM109, but remained sensitive to other classes of antibiotics (kanamycin, ciprofloxacin, tetracycline, and chloramphenicol) (Table 1). After 14 passages in media with or without 16 µg/mL of ceftazidime, A. lwoffii SGC-HZ9 and the transformant JM109-SGC-HZ9 stably maintained the blaNDM-1-containing plasmid. However, transconjugant J53-SGC-HZ9 lost blaNDM-1 within three passages when grown in antibiotic-free media, but maintained blaNDM-1 in the presence of ceftazidime.

Genetic sequence flanking blaNDM-1 in pAL-01

Following DNA cloning and modified random primer sequence walking analysis, a 6.5 kb region surrounding the blaNDM-1 gene (GenBank Accession No. JN616388) was obtained. The gene region included two antimicrobial resistance genes (blaNDM-1 and aphA6), three mobile elements (IS911, issB, and IS911-128), and phosphorosylalanlanilinase isomerase gene trpF. A comparison of the regions surrounding blaNDM-1 in the plasmids of E. coli p271A, A. lwoffii pAL-01, K. pneumoniae pKPANDM-1, and E. coli pNDM-HK is presented in Figure 2. The 2022 bp blaNDM-1-containing region in pAL-01 shared 99% sequence identity with the corresponding region in pKPANDM-1 from K. pneumoniae 05-506 (GenBank no. FN396576), with an additional 24 bp inserted into the region between blaoM-1 and trpF in pAL-01. This additional 24 bp was also found in pA271A and pNDM-HK [12,13].

An intact insertion sequence (IS) element, IS911-128, flanked by imperfect terminal repeats (IR-1, AAACCTTGAAGTGGCACA, IR-R GTGCTGACCTCTATGTTT) was identified immediately upstream of the blaoM-1 gene. This IS element belongs to the widely distributed IS30 family and was previously identified in A. baumannii (GenBank no. AV515333), where it is associated with insertional inactivation of an outer-membrane porin [14]. Notably, the blaNDM-1 upstream regions in pKPANDM-1, p271A, and pNDM-HK only contained partial IS911-128 sequences, along with the terminal repeat IR-R. In these examples, the IS element was truncated by other insertion sequences, including IS3200 in pA271A and IS26 in pKPANDM-1 and pNDM-HK (Figure 2). Interestingly, the previously confirmed −35 (TTGAAAT) blaNDM-1 promoter sequence [12] overlaps with the inverted repeat IR-R of IS911-128, and the nucleotides TGA in the −35 box form the terminal codon of IS911-128 (Figure 2).

Upstream of IS911-128 is an open reading frame encoding an aminoglycoside phosphotransferase, which is 98.5% similar to the kanamycin resistance gene aphA6 in A. baumannii (GenBank no. P09885). Further upstream are two adjacent mobile genes that are identical to putative transposable OrtB (namely issB) in A. baumannii 1656-2 (GenBank no. CP001921), and the IS3/IS911 family transposase previously identified in A. baumannii (GenBank no. CP001921). Downstream of blaoM-1, the phosphorosylalanlanilinase isomerase gene trpF (639 bp) shows 100% similarity to the first 581 nucleotides of trpF in pNDM-HK and pKPANDM-1 (Figure 2). The TrpF protein shares 65% amino acid similarity with the phosphorosylalanlanilinase isomerase of Erythrobacter sp. SD-21 (ZP_01863115).

**Discussion**

Nine Gram-negative non-fermentative bacteria (three C. indolgenes, three S. maltophilia, two Myroides spp, and one A. lwoffii) with low susceptibility to imipenem were identified in this study. Acinetobacter are common, free-living saprophytes found in soil, water, sewage, and animal source foods. It has been reported that Acinetobacter species constitute up to 22-7% of the total microflora of chicken carcasses [15]. A. lwoffii is one of the most predominant
species of Acinetobacter involved in economically-important spoilage of foods such as bacon, chicken, eggs, and fish, even when stored under refrigerated conditions or following irradiation treatment [15]. In the past, Acinetobacter spp. were regarded as saprophytes of little clinical significance. However, with the introduction of powerful new antibiotics in clinical practice and agriculture, and the use of invasive procedures in hospital intensive care units, antibiotic resistance-related community- and hospital-acquired Acinetobacter infections have emerged with increasing frequency [16,17].

All nine Gram-negative species isolated in this study were positive for a MBL phenotype. However, only four were found to contain known MBL-conferring genes. Importantly, the newly discovered MBL gene \( \text{bla} \) was identified in \( A. \ lwoffii \) SGC-HZ9, which not only presented high MIC values to nearly all \( \beta \)-lactams, but was also resistant to four other classes of antibiotics (kanamycin, ciprofloxacin, tetracycline, and chloramphenicol). This isolate was derived from the anal swab of a chicken. Although information on antimicrobial therapy for this particular chicken was not available, the antibiotic usage records for the chicken farm indicated that a number of antimicrobial agents, including penicillin, cefradine, cefadroxil, tilmicosin, doxycycline, and neomycin had been used for curing or preventing bacterial infections. To the best of our knowledge, this is the first report of the \( \text{bla} \) gene in \( A. \ lwoffii \) of food animal origin, which could have a serious public health implication because the species of \( A. \ lwoffii \) is a ubiquitous bacterium in foodstuffs, and can be transferred to human by the food chain. In addition, two Myroides spp. isolates derived from the anal swab of a pig harbored \( \text{bla} \), an intrinsic chromosomal MBL gene from \( M. \ odoratimimus \) that encodes a MUS-1 subgroup 3a metalloenzyme [18]. Despite these findings, six MBL-positive isolates (three \( C. \ indologenes \) and three \( S. \ maltophilia \)) did not contain known intrinsic MBL genes \( \text{bla} \) or \( \text{bla} \) as determined by PCR amplification. This might be due to the molecular heterogeneity of MBL genes in these species and requires further investigation.

Although the use of carbapenem antibiotics in food producing animals is prohibited in China and other countries, other \( \beta \)-lactam antibiotics such as penicillin and cephalosporins (cefadroxil, cefotaxim, and cefotaxime) have been extensively used in animal agriculture for the prevention and control of disease. Use of these

<table>
<thead>
<tr>
<th>Table 1. Antimicrobial susceptibility patterns of ( A. \ lwoffii ) SGC-HZ9, transformants and transconjugants.</th>
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<tbody>
<tr>
<td><strong>Antimicrobial agents</strong></td>
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<td>--------------------------</td>
</tr>
<tr>
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</tr>
<tr>
<td>KAN</td>
</tr>
<tr>
<td>GEN</td>
</tr>
<tr>
<td>CHL</td>
</tr>
</tbody>
</table>

*IMP, Imipenem; MER, Meropenem; ERT, Ertapenem; AMP, Ampicillin; CRA, Cefradine; COT, Cefotaxime; CAZ, Cefazadine; CEP, Cefepime; AZT, Aztreonam; POL, Polymyxin B; CIP, Ciprofloxacin; TET, Tetracycline; KAN, Kanamycin; GEN, Gentamicin; CHL, Chloramphenicol.

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Figure 1. Identification of a mobile \( \text{bla} \) gene. (A) PFGE analysis of the plasmid preparations. (B) Southern hybridization of the PFGE gel and S1 nuclease PFGE gel with a \( \text{bla} \) probe. For both panels, lanes 1–3 contain plasmids prepared from \( A. \ lwoffii \) SGC-HZ9, transformant JM109-SGC-HZ9, and transconjugant J53-SGC-HZ9, respectively. M: Lambda Ladder PFGE Marker (NEB, UK).

doi:10.1371/journal.pone.0037152.g001
non-carbapenem β-lactams in animal production may serve as a driving force for selecting MBL genes in bacteria. However, owing to the co-existence of MBL genes and other antimicrobial resistance genes, such as the \( \text{aph} \, \text{A}6 \) in \( \text{pAL-01} \) identified in this study, the possibility of cross-selection of MBL genes through use of other antimicrobials cannot be ignored. It should be noted that some carbapenemase-positive Gram-negative bacteria, including Enterobacteriaceae, with MIC values lower than \( 8 \mu \text{g/mL} \) might have been missed under the conditions used in this study.

Plasmid \( \text{pAL-01} \) was successfully transferred from SGC-HZ9 to \( \text{E. coli} \) \( \text{J55} \) using filter mating and to \( \text{E. coli} \) \( \text{JM109} \) using electroproporation, suggesting that this plasmid is mobile. The transformant and transconjugant were resistant to or showed decreased susceptibility to almost all tested β-lactams, but remained sensitive to other tested classes of antibiotics. These findings confirm the function of \( \text{pAL-01} \) in conferring resistance to β-lactams, and suggest that the resistance to non-β-lactam antibiotics in SGC-HZ9 may be due to a chromosomal gene or plasmid other than \( \text{pAL-01} \). Interestingly, the \( \text{bla}_{\text{NDM-1}} \)-harboring plasmid was highly stable in \( \text{A. lwoffii} \) \( \text{SGC-HZ9} \) and transformant \( \text{JM109-SGC-HZ9} \), but was lost in transconjugant \( \text{J53-SGC-HZ9} \) (Fig. 2), and given that an intact \( \text{IS}_{\text{Ab125}} \) has not been found in bacterial species other than \( \text{Acinetobacter} \), it is possible that the \( \text{bla}_{\text{NDM-1}} \) gene may have originated in \( \text{Acinetobacter} \), one of the most ubiquitous bacterial species in the environment. Like \( \text{bla}_{\text{NDM-1}} \), the \( \text{aph} \, \text{A}6 \) gene was found to be located immediately upstream of \( \text{IS}_{\text{Ab125}} \) (Fig. 2). However, transferring the \( \text{aphA6} \) gene (carried on \( \text{pAL-01} \)) to \( \text{E. coli} \) did not alter its susceptibility to kanamycin. It is therefore likely that \( \text{aphA6} \) in \( \text{pAL-01} \) was not expressed (or expressed at an insignificant level) in the \( \text{E. coli} \) host. Alternatively, the high MIC value of kanamycin in \( \text{SGC-HZ9} \) (64 \( \mu \text{g/mL} \)) might be conferred by other resistance determinants.

The results of this study confirm that \( \text{bla}_{\text{NDM-1}} \) and other MBL genes (\( \text{bla}_{\text{MUC-1}} \)) are present in bacteria of food animal origin. These findings have an important implication for the dissemination of this resistance gene, as \( \text{Acinetobacter} \) spp. are highly prevalent in food-producing animals (chickens and pigs) and in the environment (soil, surface water, and sewage). It would be difficult to contain the spread of \( \text{bla}_{\text{NDM-1}} \) if it can be transferred to humans via the food chain. However, it is unclear whether the \( \text{bla}_{\text{NDM-1}} \)-carrying \( \text{A. lwoffii} \) strain of food animal origin identified in this study represents a sporadic case or the early stage of a wide dissemination trend. Therefore, enhanced and continued efforts are needed to monitor the prevalence of \( \text{bla}_{\text{NDM-1}} \) in \( \text{Acinetobacter} \) spp. and other bacterial species of food animal origin.

Materials and Methods

Collection and identification of bacteria with low susceptibility to imipenem

A total of 396 samples were collected from eight chicken farms \( (n = 146, \text{cloacal swabs}) \), six duck farms \( (n = 50, \text{cloacal swabs}) \), one pig farm \( (n = 70, \text{rectal swabs}) \), and one pig slaughterhouse \( (\text{rectal swabs} \, n = 60 \text{ and nasal swabs} \, n = 50) \) from the carcasses before...
and was used in the PCR assay as a positive control for Institution (BGI, Beijing, China) according to the published bla coding sequence of listed in Table 2. All PCR amplicons were sequenced. The entire Primers for mobile MBL gene bla genes with 15% glycerol until testing. All collected strains were stored at 16 s rDNA gene, using previously described primers [25]. Gram- and identified using Gram staining and sequence analysis of the Conjugation and transformation experiments negative bacteria Phenotypic and molecular detection of MBL in Gram-


doi:10.1371/journal.pone.0037152.t002

Table 2. MBL gene primers designed in this study.

<table>
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<th>Primers</th>
<th>Sequence</th>
<th>GenBank accession No.</th>
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<tr>
<td>KHM-F</td>
<td>AATGGTAGGGTGTGTATGGA</td>
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<tr>
<td>KHM-R</td>
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<td>Chromosomal MBL</td>
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Plasmid stability
Plasmid stability was assessed by serial passage of SGC-HZ9, transconjugant J53-SGC-HZ9, and transformant JM109-SGC-HZ9 on antibiotic-free (16 µg/mL) LB media. Carriage of blaNDM-1 in these strains was assessed by inoculating the cultures of different passages on LB plates containing 16 µg/mL ceftazidime. Additionally, presence of blaNDM-1-carrying plasmids in cultures of different passages was confirmed by PCR assay. A plasmid was designated unstable if it was lost after three consecutive passages in antibiotic-free media.

DNA cloning, sequencing, and analysis
Cloning was performed using vector pHSG298 with a kanamycin resistance marker (Takara, Dalian, China). Restriction endonucleases EcoRI, XbaI, and BamHI (NEB, UK) were used to digest the plasmid DNA extracted from A. lwoffii SGC-HZ9 and transformant JM109-SGC-HZ9. Fragments were then separately ligated into pHSG298, which had been previously digested with the same enzymes. Transformation was carried out using electroporation into E. coli JM109. Transformants were selected on LB agar plates containing ceftazidime (16 µg/mL) and kanamycin (50 µg/mL). Recombinant plasmids containing blaNDM-1-positive inserts were confirmed by sequencing. The flanking regions of blaNDM-1 carrying restriction fragments in the plasmid of SGC-HZ9 were further sequenced using a modified random primer sequence walking strategy, as described previously [31]. The obtained sequences were annotated using the VectorNTI program (Invitrogen, Carlsbad; CA, USA), and sequence comparison was performed using BLASTP and BLASTN.
**Author Contributions**
Conceived and designed the experiments: Yang Wang CW QZ JS. Performed the experiments: Yu Wang JQ HL TH LM JL. Analyzed the data: CW ZS YL. Contributed reagents/materials/analysis tools: HL TH LM JL. Wrote the paper: Yang Wang QZ JS.

**References**