RESEARCH ARTICLE

Molecular characterization of \( \text{bla}_{\text{KHM-1}} \) encoding plasmid in an \textit{Enterobacter hormaechei} subsp. \textit{hoffmannii} isolate from blood culture

Kaoru Umeda, Hiromi Nakamura, Akira Fukuda, Takahiro Yamaguchi, Yuki Matsumoto, Daisuke Motooka, Shota Nakamura, Ryuji Kawahara

1 Division of Microbiology, Osaka Institute of Public Health, Osaka, Japan, 2 Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

*kaor-umeda@iph.osaka.jp

Abstract

KHM-1 was first reported in 1997 in Japan as a novel metallo-\( \beta \)-lactamase mediated by \textit{Citrobacter freundii} carrying pKHM-1 plasmid. There have been few reports in the clinical field since then. A \( \text{bla}_{\text{KHM-1}} \)-positive \textit{Enterobacter hormaechei} subsp. \textit{hoffmannii} in \textit{E. cloacae} complex, isolate OIPH-N069 was isolated from an inpatient blood culture in 2016. The isolate was characterized by whole-genome sequencing, comparative analysis of the \( \text{bla}_{\text{KHM-1}} \) encoding plasmid, antimicrobial susceptibility tests, and bacterial conjugation. OIPH-N069 was classified into ST78 of \textit{E. cloacae} complex, and was multidrug resistant because of the presence of antimicrobial resistance genes in addition to \( \text{bla}_{\text{KHM-1}} \) on its chromosome and plasmids. \( \text{bla}_{\text{KHM-1}} \) was located on 136,816 bp of the IncA/C\(_2\) plasmid pN069-1, which could be transferred to different bacterial species. The backbone structure, genetic arrangement of the class 1 integron cassette, and the \( \text{bla}_{\text{KHM-1}} \) gene located downstream of the IncA/C\(_2\) antibiotic resistance island, ARI-A, in pN069-1 and pKHM-1 were identical. Horizontal gene transfer of the \( \text{bla}_{\text{CTX-M-2}}\)–\( \text{IS}_{\text{Ecp1}} \) resistance gene module only occurred with pN069-1. The study findings indicate not only the structural conservation of \( \text{bla}_{\text{KHM-1}} \) encoding plasmids over time and across species, but also the risk of the spread of \( \text{bla}_{\text{KHM-1}} \) encoding plasmids to other bacterial species and the accumulation of additional resistance genes.

Introduction

The spread of carbapenemase-producing \textit{Enterobacteriaceae} (CPE) is a serious public health concern [1, 2]. The effectiveness of antibiotics against CPE is limited because carbapenemases can hydrolyze many \( \beta \)-lactams. In addition, CPE isolates are often resistant to other types of antimicrobial agents [2, 3]. The Ambler classification system includes three classes of carbapenemases, class A includes KPC and GES, class B is metallo-\( \beta \)-lactamases including VIM, IMP and NDM, and class D includes OXA-48 [1]. The predominant carbapenemases differ by country and region [1].
KHM-1 was firstly reported as a metallo-β-lactamase produced by *Citrobacter freundii* strain KHM243 isolated in 1997 in a Japanese hospital from a patient with a catheter-associated urinary tract infection [4]. This strain is resistant to most β-lactams other than monobactams. The *bla*KHM-1 gene includes a 726-bp open reading frame located on a transferable plasmid and encodes 241 amino acids. The protein has 59% identity with the IMP-1 and SIM-1 metallo-β-lactamases. Since then, there have been few reports on *Enterobacteriaceae* harboring *bla*KHM-1 in the clinical field.

Plasmids carrying carbapenemase genes play an important role in the spread of resistance genes to different clones and bacterial species [2]. The plasmids have shown diverse replicon types, and usually have carried carbapenemase genes on a mobile genetic element such as a transposase or insertion sequence [1, 2]. These emphasize the detailed analysis of the plasmids harboring carbapenemase genes can help to understand the mechanism of gene acquisition and to trace the route of transmission.

This study describes an isolate of metallo-beta-lactamase producing and *bla*KHM-1-positive *Enterobacter hormaechei* subsp. *hoffmannii* OIPH-N069 isolated from a blood culture in Osaka, Japan in 2016. *E. hormaechei* subsp. *hoffmannii* is one of the five subspecies of *E. hormaechei* included in *E. cloacae* complex [5]. To molecular analysis of this isolates, we performed whole-genome sequencing (WGS), comparative analysis of *bla*KHM-1 encoded plasmids, antimicrobial susceptibility tests and bacterial conjugation.

**Materials and methods**

**Bacterial isolate**

Isolate OIPH-N069 was isolated in 2016 from the blood culture of a hospital inpatient in Osaka, Japan. The isolate was identified as a carbapenem-resistant *Enterobacter cloacae* complex at the hospital laboratory. The biochemical profile was evaluated with an API 20E microorganism identification kit (Sysmex bioMerieux).

**Antibiotic susceptibility testing**

The minimal inhibitory concentrations (MICs) for piperacillin, cefmetazole, cefoxitin, ceftazidime, cefotaxime, cefpodoxime, aztreonam, imipenem, meropenem, gentamicin, amikacin, nalidixic acid, ciprofloxacin, and trimethoprim-sulfamethoxazole were assayed by the Dry Plate EIKEN test (Eiken Chemical). The MIC for fosfomycin (Wako) was evaluated by agar dilution, and the MICs for colistin (Wako), piperacillin/tazobactam (Tokyo Chemical Industry), and tigecycline (Sigma-Aldrich) were assayed by broth dilution according to CLSI document M100-S25 performance standards [6]. *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as controls.

**Phenotypic and genetic analysis of carbapenem resistance**

Carbapenemase production was confirmed by the Carba NP test II [7] and double disk synergy test using sodium mercaptoacetic acid (SMA) disks (Eiken Chemical) as an inhibitor. The detection of carbapenemase metallo-β-lactamase genes (*bla*KHM-1, *bla*IMP, *bla*VIM, *bla*NDM, *bla*KPC, *bla*OXA-48, *bla*SMB, and *bla*GES) was performed by polymerase chain reaction (PCR) [8].

**Bacterial conjugation**

Transfer of the *bla*KHM-1 carrying plasmid was confirmed by a filter-mating method described by Kudo [9] with slight modifications. Briefly, the recipient was a rifampicin-resistant...
Escherichia coli K-12 DH5α strain [10]. The donor, recipient, and transconjugant were selected on MacConkey agar (OXOID) supplemented with 0.5 μg/mL meropenem (Wako) and 50 μg/mL rifampicin (Wako) [11]. Colonies were counted after overnight incubation at 37°C. The transfer frequency was reported as the ratio of the numbers of transconjugant to recipient colonies (transconjugant/recipient). The procedure was repeated in triplicate. The transconjugant was tested for antimicrobial sensitivity, and the presence of carbapenemase was confirmed by the CarbaNP test II and PCR of blaKHM-1 as described above.

S1-nuclease digested pulsed-field gel electrophoresis (S1-PFGE) and Southern blot hybridization

Agarose gel plugs were prepared by culturing isolates at 37°C for 18 h in trypto-soya broth (Nissui Pharmaceutical) and SeaKem Gold agarose (Lonza). Plugs were treated with 1 mg/ml proteinase K (Sigma-Aldrich), digested with 18 U of S1 nuclease (Takara Bio), and electrophoresed on a CHEF-DRIII apparatus (Bio-Rad Laboratories) in 1% SeaKem Gold agarose at 14°C and 6 V/cm for 17 h with switching times of from 2.2 to 54.2 s. The Salmonella enterica serovar Braenderup strain H9812 digested with XbaI (Roche Diagnostics) was used as a marker for PFGE. DNA fragments were visualized by ethidium bromide staining and then transferred to Hybond N+ nylon membranes (GE Healthcare) using a capillary transfer system. Hybridization was carried out with a digoxigenin (DIG)-labeled blaKHM-1 probe prepared from DNA extracted from isolate OIPH-N069 using a KHM-F/KHM-R primer pair [8] and a PCR DIG probe synthesis kit (Roche Diagnostics). Hybridization signals were detected with a DIG luminescent detection kit (Roche Diagnostics) and an Amersham Image 600 (GE Healthcare).

WGS and plasmid sequencing

Genomic DNAs were sheared to 600 bp segments with a focused-ultrasonicator S220 (Covaris) to prepare a short-read sequencing library. DNA was sheared to 8,000 bp segments for long-read sequencing with g-TUBE (Covaris). The short-read library was prepared with dual-indexed, 300 bp paired-end reads using MiSeq Reagent v3 kits (600 cycles) and sequenced with an MiSeq instrument (Illumina). The long-read library was prepared using Ligation Sequencing 1D kits and sequenced with an MinION sequencer (Oxford Nanopore Technologies). Genomic assembly of both short and long reads was performed with Unicycler v0.4.4 [12].

The genome sequences were annotated using the DNA Data Bank of Japan (DDBJ) Fast Annotation and Submission Tool (DFAST, https://dfast.nig.ac.jp/) as described by Tanizawa [13]. In the plasmid analysis, antibiotic resistance genes were identified using ResFinder 3.1 [14], plasmid incompatibility replicon typing was performed with PlasmidFinder 2.0 [15] and multilocus sequence typing (MLST) was performed with MLST 2.0 [16], on the Center for Genomic Epidemiology website (http://www.genomicepidemiology.org/). The bacterial species was identified using the ANI calculator (http://enve-omics.ce.gatech.edu/ani/) [17]. The species identification cut off was 95%–96% of the and the subspecies cutoff was 98% compared with the genomic sequences of the type strain [5, 18, 19]. The insertion sequence element was identified by IS finder (https://www-is.biotoul.fr/index.php). The integron ID was retrieved from the INTEGRALL database (http://integrall.bio.ua.pt/) [20]. Circular representations of plasmid sequences were visualized using the BLAST Ring Image Generator (BRIG, http://brig.sourceforge.net) [21]. The plasmid structure was generated in Easyfig 2.2.3 (https://mjsull.github.io/Easyfig/) [22].
Nucleotide sequence accession numbers

The complete, annotated genomic sequences were submitted to The DDBJ with the accession numbers AP019817 (chromosome), AP019818 (pN069-1), AP019819 (pN069-2), AP019820 (pN069-3), and AP019821 (pN069-4), associated with BioProject accession number PRJD8177 and Biosample number SAMD00167169.

Ethical statement

The study was approved by the ethical review committee of Osaka Institute of Public Health (approval No. 1402-04-3).

Results

Antimicrobial susceptibilities and detection of blaKHM-1

The antimicrobial susceptibilities of 18 antibiotics to the OIPH-N069 isolate are shown in Table 1. For β-lactams, this isolate showed resistance to piperacillin, cefmetazole, cefoxitin, ceftazidine, cefotaxime, cefpodoxime, aztreonam and meropenem, and showed intermediate resistance to imipenem and piperacillin/tazobactam. Other, it also showed resistance to trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin and tigecycline. But, it was susceptible to fosfomycin, aminoglycosides and colistin. The OIPH-N069 isolate was a metallo-β-lactamase producer, positive in both the Carba NP test II and the SMA double disk synergy test. PCR screening of carbapenemase genes detected only blaKHM-1.

Conjugation assay

The conjugation procedure used the OIPH-N069 isolate as the donor and the rifampicin-resistant E. coli K-12 DH5α strain as the recipient. Transconjugants positive for Carba NP II and blaKHM-1 by PCR were successfully obtained following selection with meropenem and rifampicin. The antimicrobial susceptibility results obtained with the transconjugant yielded MICs similar or identical to those for the OIPH-N069 isolate, except for lower MICs for aztreonam, fosfomycin, quinolones, trimethoprim-sulfamethoxazole, piperacillin/tazobactam, and tigecycline than those for OIPH-N069 (Table 1). The transfer frequency was 2.9 × 10^{-7}.

S1-PFGE and Southern blot hybridization

The S1-PFGE results showed that the OIPH-N069 isolate carried three plasmids in addition to the bacterial chromosome. The TcN069 transconjugant carried two plasmids from the OIPH-N069 isolate (Fig 1A). Southern blot hybridization found that blaKHM-1 was located on a plasmid with approximately 130 kbp in both the OIPH-N069 isolate and the TcN069 transconjugant (Fig 1B).

WGS analysis of the OIPH-N069 isolate

The genome sequences of the OIPH-N069 isolate are shown in Table 2. The OIPH-N069 genome comprised a chromosome and four plasmids and carried nine antimicrobial resistance genes. The genome included chromosomal sequences that incuded 4,689,117 bp identified as E. hormaechei subsp. hoffmannii by the average nucleotide identity (ANI) value compared with the genomic sequences of the type strains of the E. cloacae complex [5, 18]. The biochemical profile of the OIPH-N069 isolate was identical to that of E. cloacae complex. The sequence type (ST) for the chromosome was ST78. The chromosome encoded antimicrobial resistance
The four plasmids included pN069-1 (136,816bp, IncA/C2, harbored aadA2, aac(6’)-Ia, blaCTX-M-2, blaKHM-1 and sul1), pN069-2 (115,150bp, IncFIB, harbored dfrA15 and sul1), pN069-3 (47,299bp, Inc type was not available) and pN069-4 (2,495bp, ColRNAI plasmid).

The lower MICs of transconjugant TcN069 than isolate OIPH-N069 for aztreonam, quinolones, fosfomycin, and trimethoprim-sulphamethoxazole could relate to blaACT-5 on chromosome, QRDR mutations on chromosome, fosA on chromosome, and dfrA on pN069-2, respectively.

Comparative analysis of blaKHM-1 on pN069-1

The results of comparative genomic analysis of blaKHM-1 encoded on plasmid pN069-1 and on pKHM-1, pM216, and pEC732 are shown in Fig 2. The pKHM-1 from C. freundii strain KHM243 (AP014939) [3] was identified as an IncA/C2 type by PlasmidFinder 2.0. The other IncA/C2 plasmids, pEC732 (74% query cover and 94.41% identity) carrying blaIMP-14 from E. coli (CP015139) [23] and pM216 (73% query cover and 94.41% identity) carrying blaNDM-4 from E. coli (AP018145) [24], were selected by their homology score following a BLAST search using the whole nucleotide sequence of pN069-1. The backbone structure of pN069-1 was conserved in pKHM-1 and the two other IncA/C2 plasmids, but variation of the antimicrobial-resistance genes and their surrounding regions was observed.

Table 1. Antibiotic susceptibility test results.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (μg/ml) / antimicrobial susceptibility*</th>
<th>Transconjugant TcN069</th>
<th>Recipient rifampicin-resistant E. coli DH5α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. hormaechei subsp. hoffmannii OIPH-N069</td>
<td>Transconjugant TcN069</td>
<td>Recipient rifampicin-resistant E. coli DH5α</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;256 / R</td>
<td>&gt;256 / R</td>
<td>1 / S</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>&gt;64 / R</td>
<td>&gt;64 / R</td>
<td>1 / S</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>&gt;64 / R</td>
<td>&gt;64 / R</td>
<td>4 / S</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;64 / R</td>
<td>&gt;64 / R</td>
<td>0.125 / S</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;128 / R</td>
<td>&gt;128 / R</td>
<td>≤0.125 / S</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>&gt;16 / R</td>
<td>&gt;16 / R</td>
<td>0.5 / S</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>16 / R</td>
<td>0.5 / S</td>
<td>0.125 / S</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>2 / I</td>
<td>2 / I</td>
<td>≤0.06 / S</td>
</tr>
<tr>
<td>Meropenem</td>
<td>8 / R</td>
<td>8 / R</td>
<td>≤0.06 / S</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.5 / S</td>
<td>0.5 / S</td>
<td>0.25 / S</td>
</tr>
<tr>
<td>Amikacin</td>
<td>1 / S</td>
<td>4 / S</td>
<td>2 / S</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>&gt;128 / R</td>
<td>4 / S</td>
<td>4 / S</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>16 / R</td>
<td>≤0.03 / S</td>
<td>≤0.03 / S</td>
</tr>
<tr>
<td>Trimethoprim-sulphamethoxazole</td>
<td>&gt;4/376 / R</td>
<td>0.12/2.38 / S</td>
<td>0.12/2.38 / S</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.25 / S</td>
<td>0.5 / S</td>
<td>0.5 / S</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>64/4 / I</td>
<td>4/4 / S</td>
<td>2/4 / S</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>1 / R</td>
<td>0.125 / S</td>
<td>0.125 / S</td>
</tr>
</tbody>
</table>

* Breakpoints of Enterobacteriaceae generated by CLSI M100-ED29:2019, except for colistin and tigecycline by EUCAST_v9.0. S, susceptible; I, intermediate; R, resistant.

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genesis of blaACT-5 and fosA, and also had mutations in quinolone resistance-determining regions (QRDR) of gyrA (Ser/Thr83 to Ile) and parC (Ser80 to Ile).

The four plasmids included pN069-1 (136,816bp, IncA/C2, harbored aadA2, aac(6’)-Ia, blaCTX-M-2, blaKHM-1 and sul1), pN069-2 (115,150bp, IncFIB, harbored dfrA15 and sul1), pN069-3 (47,299bp, Inc type was not available) and pN069-4 (2,495bp, ColRNAI plasmid).

The lower MICs of transconjugant TcN069 than isolate OIPH-N069 for aztreonam, quinolones, fosfomycin, and trimethoprim-sulphamethoxazole could relate to blaACT-5 on chromosome, QRDR mutations on chromosome, fosA on chromosome, and dfrA on pN069-2, respectively.
Fig 1. S1-PFGE (A) and Southern blot hybridization detection (B) of \textit{bla}\textsubscript{KHM-1}. Lane 1, \textit{E. hormaechei} subsp. \textit{hoffmannii} isolate OIPH-N069; Lane 2, transconjugant TcN069; Lane 3, \textit{E. coli} K-12 DH5\(\alpha\) (recipient); Lane M; Xba I-digested \textit{Salmonella enterica} serovar Braenderup strain H9812.

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Genetic structure of antimicrobial resistance regions

The genetic structure of the antimicrobial resistance region in pN069-1 was compared with that in pKHM-1 (Fig 3). This region contained several transposase/recombinase genes. The \textit{bla}\textsubscript{KHM-1} nucleotide sequences (100% identity) and the gene arrangements of its downstream containing lyoxalase family and hypothetical proteins were well conserved in both plasmids. The class 1 integron cassette, its surrounding transposases containing Tn3 and IS91 family protein genes, and the \textit{mer}-operon were also homologous in both plasmids. The genetic

![Genetic structure of antimicrobial resistance regions](https://do i.org/10.1371/j ournal.pone.0227605.g002)

Table 2. Whole genome information for \textit{E. hormaechei} subsp. \textit{hoffmannii} isolate OIPH-N069.

<table>
<thead>
<tr>
<th>Replicon</th>
<th>Nucleotide length (bp)</th>
<th>Number of coding sequence</th>
<th>GC%</th>
<th>Inc type</th>
<th>Antimicrobial resistance genes</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome</td>
<td>4,689,117</td>
<td>4,375</td>
<td>55.2</td>
<td>NA</td>
<td>\textit{bla}\textsubscript{ACT-5}, fosA</td>
<td>AP019817</td>
</tr>
<tr>
<td>pN069-1</td>
<td>136,816</td>
<td>163</td>
<td>50.1</td>
<td>IncA/C\textsubscript{2}</td>
<td>\textit{aadA2}, \textit{aac}(6')-\textit{lae}, \textit{bla}\textsubscript{CTX-M-1}, \textit{bla}\textsubscript{KHM-1}, \textit{sul1}</td>
<td>AP019818</td>
</tr>
<tr>
<td>pN069-2</td>
<td>115,150</td>
<td>139</td>
<td>51.8</td>
<td>IncFIB</td>
<td>\textit{dfrA15}, \textit{sul1}</td>
<td>AP019819</td>
</tr>
<tr>
<td>pN069-3</td>
<td>47,299</td>
<td>63</td>
<td>51.1</td>
<td>NA</td>
<td>ND</td>
<td>AP019820</td>
</tr>
<tr>
<td>pN069-4</td>
<td>2,495</td>
<td>3</td>
<td>51.5</td>
<td>ColRNAI</td>
<td>ND</td>
<td>AP019821</td>
</tr>
</tbody>
</table>

NA, not available; ND, not detected.

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![Fig 2. Circular representation of pN069-1.](https://do i.org/10.1371/j ournal.pone.0227605.g002)

Genetic structure of antimicrobial resistance regions

The genetic structure of the antimicrobial resistance region in pN069-1 was compared with that in pKHM-1 (Fig 3). This region contained several transposase/recombinase genes. The \textit{bla}\textsubscript{KHM-1} nucleotide sequences (100% identity) and the gene arrangements of its downstream containing lyoxalase family and hypothetical proteins were well conserved in both plasmids. The class 1 integron cassette, its surrounding transposases containing Tn3 and IS91 family protein genes, and the \textit{mer}-operon were also homologous in both plasmids. The genetic

![Genetic structure of antimicrobial resistance regions](https://do i.org/10.1371/j ournal.pone.0227605.g002)
structure of class 1 integron cassette \([\text{int1}, \text{aadA2}, \text{aac(6)-lae}, \text{qacEdelta1}, \text{and sul1}]\) was not found in GenBank or the INTEGRALL database. The IS5 family transposase (identified as IS\(_{Ec68}\)) located just upstream of \(\text{bla}^{\text{KHM-1}}\), \(\text{bla}^{\text{CTX-M-2}}\) with the tnpA family transposase (identified as IS\(_{Ecp1}\)) were found only in pN069-1. Fourteen coding sequences containing IS66 and IS1634 family transposases located approximately 9.2 kbp upstream of \(\text{bla}^{\text{KHM-1}}\) were found only in pKHM-1.

**Discussion**

In this study, we analyzed a KHM-1-producing \(E.\) hormaechei subsp. hoffmannii isolate OIPH-N069 from the blood sample of an inpatient. \(E.\) hormaechei is the most frequently identified in clinical isolates of \(E.\) cloacae complex member [5], and ST78 is one of the most common clone among multidrug- or carbapenem-resistant isolates in worldwide [25] including in Japan [26]. OIPH-N069 was isolated 19 years after the \(C.\) freundii KHM243 strain [4], but the backbone structure of plasmids and peripheral structure of \(\text{bla}^{\text{KHM-1}}\) were relatively well conserved, even with the presence of some deletions and insertions (Figs 2 and 3). Although the \(\text{bla}^{\text{KHM-1}}\) encoding plasmid has rarely found, it may have been repeatedly transferred to multiple bacterial species and maintained for many years. The results of conjugation assay (Fig 1) also suggest that the \(\text{bla}^{\text{KHM-1}}\) encoding IncA/C\(_2\) plasmid can spread to other species. Just recently, KHM-1–producing \(Klebsiella\) quasipneumoniae subsp. quasipneumoniae was isolated from wastewater in Tokyo, Japan [27]. Ongoing comprehensive surveillance with characterization of antimicrobial resistance plasmids is recommended to monitor the spread and resistance of KHM-1–producing \(Enterobacteriaceae\) in clinical and other settings.

OIPH-N069 showed resistance to several kinds of antimicrobials, and that corresponded to antimicrobial resistant genes on its chromosome and plasmids (Tables 1 and 2). OIPH-N069 was found to be a multidrug-resistant \(Enterobacteriaceae\) according to the international standard criteria described by Magiorakos et al. [28], and more resistant than \(C.\) freundii KHM243.
such as aztreonam and tigecycline, which are used to treat infections caused by carbapenem-resistant Enterobacteriaceae [3, 4] (Table 1).

The antibiotic resistance island of the IncA/C$_2$ plasmid located upstream of the rhs gene is called as the ARI-A region [29, 30], but bla$_{KHM-1}$ was located downstream of the ARI-A island, both in pN069-1 and pKHM-1 (Figs 2 and 3). The IS5 family transposase ISEc68 was found just upstream of bla$_{KHM-1}$ in pN069-1, but pKHM-1 had another transposases. So that, the origin of bla$_{KHM-1}$ and how it came to be inserted in this region could not be determined. The class 1 integron cassette in the pN069-1 harbored resistance genes of aadA2, aac(6')-lae and sul1, homologous with pKHM-1, and also the other IncA/C$_2$ plasmids except for aac(6')-lae. The bla$_{CTX-M-2}$ was present only in the ARI-A region of pN069-1 (Figs 2 and 3). The bla$_{CTX-M-2}$-ISEcp1 module has been reported in antimicrobial resistance plasmids from other species, for example, the multidrug resistance IncN plasmid pKPI-6 from Klebsiella pneumoniae (AB616660) epidemic in Japan [31]. It suggests the horizontal acquisition of ISEcp1-mediated bla$_{CTX-M-2}$ as a new resistance gene in pN069-1. As the ARI-A region of the IncA/C$_2$ plasmid is a reservoir of resistance genes [29, 30], the bla$_{KHM-1}$-encoding plasmid may become increasingly resistant in the future.

In conclusion, molecular characterization and WGS of an E. hormaechei subsp. hoffmannii ST78 isolate producing KHM-1 metallo-β-lactamase confirmed not only the structural conservation of bla$_{KHM-1}$-encoding plasmids over time and across species, but also that the bla$_{KHM-1}$-encoding plasmid can spread to other Enterobacteriaceae species and horizontally acquire microbial resistance genes. In the clinical aspect, antimicrobial treatment of infections caused by KHM-1-producing Enterobacteriaceae must be careful due to the possibility for MDR with multiple resistant genes in addition to bla$_{KHM-1}$.

Supporting information
S1 Fig. Original images for gel and blot. S1-PFGE gel image for Fig 1A and Southern blot hybridization image for Fig 1B were represented.
(PDF)

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Author Contributions

Conceptualization: Kaoru Umeda, Ryuji Kawahara.

Data curation: Yuki Matsumoto, Daisuke Motooka, Shota Nakamura.

Formal analysis: Yuki Matsumoto, Daisuke Motooka, Shota Nakamura.

Investigation: Kaoru Umeda, Hiromi Nakamura, Akira Fukuda.

Resources: Kaoru Umeda, Hiromi Nakamura.

Supervision: Ryuji Kawahara.

Visualization: Kaoru Umeda, Takahiro Yamaguchi.

Writing – original draft: Kaoru Umeda.

Writing – review & editing: Akira Fukuda, Ryuji Kawahara.
References


