Escherichia Coli Isolate From Hospital Sewage Is Carrying bla_{NDM-1} and bla_{oxa-10}

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Research Article

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Abstract

Carbapenems, as the “last line of defense” against gram-negative bacteria, are increasingly being challenged by drug-resistant bacteria, especially in *Enterobacteriaceae*. In this study, a carbapenems resistant gram-negative bacterium, named AH001, was isolated from hospital sewage, and modified Hoge test confirmed this bacterium can produce carbapenemase. Further analysis revealed that this bacterium is multi-drug resistance, which against additional seven antibiotics. Whole-genome sequencing and analysis showed that AH001 could not be classified by existing MLST, and its serotype couldn’t be distinguished among O9, O89 or O168 in O antigen prediction. More attention should be paid to the role of environmental source *Escherichia coli* in the development and transfer of drug resistance in the hospital environment.

1. Introduction

Among β-lactam antibiotics, carbapenem antibiotics are considered to be the “last line of defense” against gram-negative bacterial infections and are used to treat bacteria produced by ESBLs (Extend-spectrum β-lactamases) bacterial infection[1]. Recently, carbapenem-resistant bacteria can infect humans and animals and spread widely around the world, posing a serious public health threat to the world[2]. In the Ambler nomenclature, carbapenemase is divided into A, B, C and D enzymes, B is a metalloenzyme, and NDM is a B enzyme[3]. Since the first report of NDM in an Indian patient in Sweden in 2008, the bla_{NDM-1} gene has been shown to be easily spread between different types of gram-negative bacteria and can spread to many countries in a short time, becoming one of the most worrying drug resistance genes in the world[4].

NDM-1 and NDM-5 are the two most common NDM variants in *Enterobacteriaceae* in China[4]. Bla_{NDM-1} gene and its variants have been widely popular among of the *Enterobacteriaceae*, the most common members are *Escherichia coli*[5], *Klebsiella pneumoniae*[6], *Enterobacter cloacae* etc.[5–7]. Bla_{OXA-10} is a CHDLs (Carbapenem-hydrolyzing class D carbapenemases) previously detected in human pathogens, usually produced by *Pseudomonas aeruginosa*. It is a narrow-spectrum enzyme and has the greatest hydrolytic capacity to penicillin[8, 9]. Initially, these enzymes were highly resistant to penicillin, but now they are more resistant to carbapenem antibiotics, which should pay more attention on it [9].

Horizontal gene transfer plays an important role in introducing new genes into micropopulation[10]. Due to the continuous discharge of feces and urine, hospital sewage is an ideal place to spread and develop antibiotic resistance genes[11]. Despite the obvious importance of hospital sewage in the development and spread of drug resistance, the presence of ESBLs and carbapenemase-resistant bacteria in hospital sewage has not been well studied. The plasmid mediated spread of antibiotic-resistant bacteria in hospital sewage is a problem with great impact on public health, ecology and economy.

Therefore, the purpose of this research is to isolate carbapenem-resistant bacteria from hospital sewage and conduct a modified Hoge test to verify whether they produce carbapenemase, conduct a conjugate

transfer test to study whether the drug-resistant plasmids are mobile, and perform genome-wide sequencing analysis (including analysis of their serotypes, ST types, and prediction of drug-resistant genes, etc.) to assess their risk of transmission to public health through the aquatic environment.

2. Methods

2.1 Bacteria and culture methods

Escherichia coli ATCC25922 is a quality control strain for drug susceptibility test; EC600 as recipient bacteria for plasmid conjugation transfer test and was donated by the Second Affiliated Hospital of Soochow University. Bacteria were routinely cultured in Luria–Bertani (LB) broth at 37℃.

2.2 Sample collection and bacterial identification

In September 2019, the sewage was collected from the sewage outlet of a hospital in eastern China, and then the drug-resistant bacteria were isolated on a meropenem resistant plate (200 μg/ml). Resistant colonies were collected and set for further researches.

2.3 Antimicrobial Susceptibility Testing

Broth dilution method was used for antimicrobial susceptibility testing of isolated bacteria, and results were interpreted according to the criteria recommended by Clinical and Laboratory Standards Institute (CLSI, 2019). A total of 10 kinds of antibiotics were selected for antibiotics sensitivity test, including chloramphenicol, fosfomycin, tetracycline, ampicillin, gentamicin, ofloxacin, cefotaxime, rifampicin polymyxin B and meropenem (HANGWEI, Hangzhou, China). Each experiment was repeated three times.

2.4 Whole Genome Sequencing

Genomic DNA was extracted with the SDS method[12]. A total amount of 1μg DNA per sample was used as input material for the DNA sample preparations. The whole genome of isolated bacteria was sequenced using Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd. The raw data obtained by sequencing (Raw Data) had a certain proportion of low-quality data. In order to ensure the accuracy and reliability of the subsequent information analysis results, the original data must be filtered to obtain valid data (Clean Data). The genome was assembled from Clean Data of each sample after quality control.

2.5.1 Antimicrobial resistance genes prediction

Use the online tool ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) to predict resistance genes from the AH001 genome.

2.5.2 Plasmid incompatibility types

Identification of plasmid incompatibility types were performed on complete sequences of plasmids via the online servic PlasmidFinder v2.0 at CGE (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

2.5.3 Moleculer serotyping

The serotype of the *E. coli* isolate was predictied by SerotypeFinder (https://cge.cbs.dtu.dk/services/SerotypeFinder/).

2.5.4 Phylogenetic analysis

MEGAX was used to construct adjacent phylogenetic trees of the whole genome sequence of isolates and other similar *E.coli*.

2.5 Carbapenemases detection

The modified Hodge test (MHT) was performed on a MHA (Mueller-Hinton Agar) plate with meropenem as substrate for the detection of carbapenemases (CLSI, 2019). Specific steps are as follows: prepare ATCC25922 suspension with 0.5 MCF in MH and dilute it with MH at 1:10. Inoculate into the MHA plate according to the drug sensitivity test, let the plate dry for 3 ~ 10 minutes, and then stick the meropenem disk in the middle of the plate. 3 ~ 5 isolates that had been cultured overnight on blood AGAR were selected by inoculation ring, and a straight line was drawn from the edge of the plate to the center during inoculation, the line must be at least 20 mm ~ 25 mm long.

2.6 Transferability of Plasmids with Carbapenems Resistance

In order to evaluate the transferability of plasmids with carbapenem resistance, EC600 (Rifampicin resistance) was chosen as the recipient bacteria. Two isolated donor strains and EC600 were cultivated to 0.4 OD, mix 50 μl of donor bacteria with 150 μl of recipient bacteria (EC600) and cultivated in an incubator at 37℃ about 18 h respectively. The cultured mixed bacteria were cultivated on the double-resistant MH plates of meropenem and rifampicin respectively. The determination method was the mixed bacteria grew colonies on the double-resistant plate.

3. Results

3.1 Bacterial isolation and identification

The draft sequencing results of the tested bacteria were uploaded to NCBI for bacterial homology comparison. The similarity rate with *E. coli* reached 97.166% and the isolate was named as AH001.

3.2 Antimicrobial Susceptibility Testing

In addition to meropenem, AH001 exhibited resistance to 7 kinds of antibiotic include chloramphenicol, fosfomycin, tetracycline, ampicillin, gentamicin, ofloxacin, and cefotaxime, expect, rifampicin and polymyxin B (Table 1). The results showed that the multi-drug resistance of *E. coli* was serious.

Table 1
AH001 susceptibility test results for ten antibiotics

Antibiotic	MIC (μg/mL)	Result	Antibiotic	MIC (μg/mL)	Result
Gentamicin	>256	R	Meropenem	>256	R
Ofloxacin	≥ 8	R	Cefotaxime	>256	R
Polymyxin B	≤ 2	S	Fosfomycin	>256	R
Chloramphenicol	≥ 128	R	Rifampicin	≤ 1	S
Ampicillin	>256	R	Tetracycline	≥ 128	R

3.3 Bioinformatics assays of genome of AH001

The results of antibiotics resistant genes prediction showed that the genome of AH001 not only contains the genes of *bla*$_{NDM-1}$ and *bla*oxa-10, but other 22 drug resistance genes (Table 2). Plasmid types include ColE10, IncC, IncHI2, IncHI2A, IncHI2A. Serotype couldn't be distinguished among O9, O89 or O168 in O antigen prediction.

After uploading the 16s rDNA sequence of AH001 to NCBI for comparison, bacteria with higher similarity were selected for evolution analysis (Fig. 1). The results show that the similarities between AH001 and different strains including *Acinetobacter baumannii* are very high.

Table 2
Predicted drug resistance gene in AH001

Antibiotic	Drug resistance gene	Contig
Aminoglycosides	*aac(3)-IV*	Scaffold60
	aac(6')-Ib3	Scaffold57
	aadA1	Scaffold57
	aadA5	Scaffold57
	aph(3")-I	Scaffold83
	aph(4)-Ia	Scaffold63
	aph(6)-Id	Scaffold60
Fosfomycin	*fosA3*	Scaffold72
Sulphonamide	*sul2*	Scaffold96
Tetracycline	*tet(A)*	Scaffold63
Trimethoprim	*dfrA14*	Scaffold57
	dfrA17	Scaffold83
Macrolide	*mdf(A)*	Scaffold2
	mph(A)	Scaffold57
Phenicol	*floR*	Scaffold73
	cmlA1	Scaffold57
Beta-lactam	bla_{CMY-2}	Scaffold33
	$bla_{CTX-M-14}$	Scaffold72
	$bla_{CTX-M-15}$	Scaffold76
	bla_{NDM-1}	Scaffold31
	bla_{OXA-10}	Scaffold57
Quinolone	*aac(6')-Ib-cr*	Scaffold57
	qnrS1	Scaffold61
	qnrVC4	Scaffold57

3.4 Carbapenemases Detection of AH001

Modified Hoge test showed it produces carbapenemase (Fig. 2).

3.5 Transferability of Plasmids

The growth of the isolate of AH001 was that there was no colony growth on the dual-resistant meropenem and rifampicin plates, which proved that the resistant plasmid was not conjugated to the recipient strain EC600.

4. Discussion

In this study, we isolated an unknown strain of *E. coli* that is resistant to meropenem from medical sewage, and then studied its resistant phenotype and genotype. It was found that it contained both bla_{NDM} and bla_{OXA-10} genes.

The emergence and rapid spread of antibiotic resistance is a growing threat to human health[13]. CRE (Carbapenem-resistant *Enterobacteriaceae*) has the ability to promote the widely spread of drug resistance genes, mainly through mobile genetic elements including natural transformation, and the process of plasmid conjugation. We found that *E. coli* AH001 is carrying NDM−1 and OXA−10, both of which are belong to carbapenem resistant enzymes. The aquatic environment, such as the sewage in hospital which are enriched both antibiotics and bacteria, is an important reservoir for drug resistance genes and can be used as a medium to spread ARGs (Antibiotic Resistance Genes) from one ecosystem to another, thereby increasing the risk of MDR (Multi-Drug Resistance) bacteria infection outside the hospital[14]. The widespread detection of CPO (Carbapenemase-producing Organism) in the environment is an emerging environmental problem that may seriously affect public health. Our results are consistent with previous studies, indicating that bla_{NDM} has appeared in many different species and spread rapidly in different environments[15, 16].

In China, a recent study of 10,273 clinical stool samples collected from 52 hospitals found that the total infection rate of intestinal bacteria carrying bla_{NDM-1} among clinical patients was 14.8%[17]. And these feces can be discharged into hospital sewage, prompting bla_{NDM-1} to spread in many species and environments[18]. In environments such as sewage, the discovery of two different carbapenemase isolates in the same strain of bacteria has become increasingly frequent[15, 19, 20].

We found the bla_{OXA-10} gene in AH001, OXA-10 type β-lactamase was previously considered a narrow-band enzyme in *E. coli* and *Pseudomonas aeruginosa*. However, Antunes NT proved that when expressed in *Acinetobacter baumannii*, this enzyme showed high resistance to carbapenems, and the production of OXA-10 increased the MIC of the bacteria to ceftazidime by 32 times[21]. These data clearly demonstrate the importance of OXA-10 as CHDLs, and how to verify that OXA-10 expresses carbapenem resistance on *E. coli* is worth exploring.

In addition to meropenem, AH001 exhibited resistance to 7 kinds of antibiotic include chloramphenicol, fosfomycin, tetracycline, ampicillin, gentamicin, ofloxacin, and cefotaxime, expect, rifampicin and polymyxin B. The results of drug susceptibility tests indicate that AH001 is severely resistant. Modified

Hoge test showed it produces carbapenemase. The results of the conjugation transfer test showed that the drug-resistant plasmid of carbapenem antibiotics mediating AH001 could not be transferred to the recipient strain EC600, and another strain of *E. coli* isolated from the sewage both carrying bla_{NDM-4} and bla_{KPC-2} was able to transfer the resistant plasmid to the recipient strain EC600. It is speculated that the reason may be that the difference between the donor bacteria and the recipient bacteria, and there are cases where the plasmids are incompatible. The AH001 isolated in this study predicted the $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$ genes by whole genome sequencing analysis. According to reports, $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$ are the major ESBL types in human clinical isolates, regardless of geographic origin[22]. It has been reported that cefotaxime resistance has been observed in patients with cephalosporin treatment of *Aeromonas* bacteremia[23].

The serotype of AH001 predicted on the CGE website showed three serotypes, namely O9, O89 and O162, and the coincidence rates were 99.31%, 94.1%, and 93.63%, respectively. It is reported that *E. coli* of the O8 serotype are commonly associated with septicemia or diarrhea in calves and pose a significant threat to the cattle industry worldwide[24]. It has also been reported that the O89: H9 serotype is related to *E. coli* that produces ESBLs and carries bla_{NDM-5} and *mcr-1* genes[25–27].

Nowadays, the detection of bacteria in the sewage is often studied by metagenomics, including 16s rDNA sequencing and the whole gene assembly of all bacteria. This study is based on direct phenotypic screening, which still has a certain significance. To the best of our knowledge, this is the first report of a carbapenem-resistant *E. coli* carrying both bla_{NDM-1} and bla_{OXA-10} genes. The sewage of hospital treatment system will continuously discharge a large number of drug resistance genes into the water environment and release it into surface water[28]. This gene can be transferred horizontally in the sewage through the MGE (Mobile Genetic Elements) carrying the gene. Therefore, more attention should be paid to the discharge of hospital sewage.

5. Conclusions

To our knowledge, this is the first report of carbapenem-resistant *E. coli* carrying both bla_{NDM-1} and bla_{OXA-10} genes. The research in this article will help us understand the *E. colii* carrying both bla_{NDM-1} and bla_{OXA-10} genes in medical sewage.

Declarations

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Conflict of interest

None declared.

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GenBank Accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JACDTS000000000. The version described in this paper is version JACDTS000000000. The SUBID is SUB7768533.

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Figures

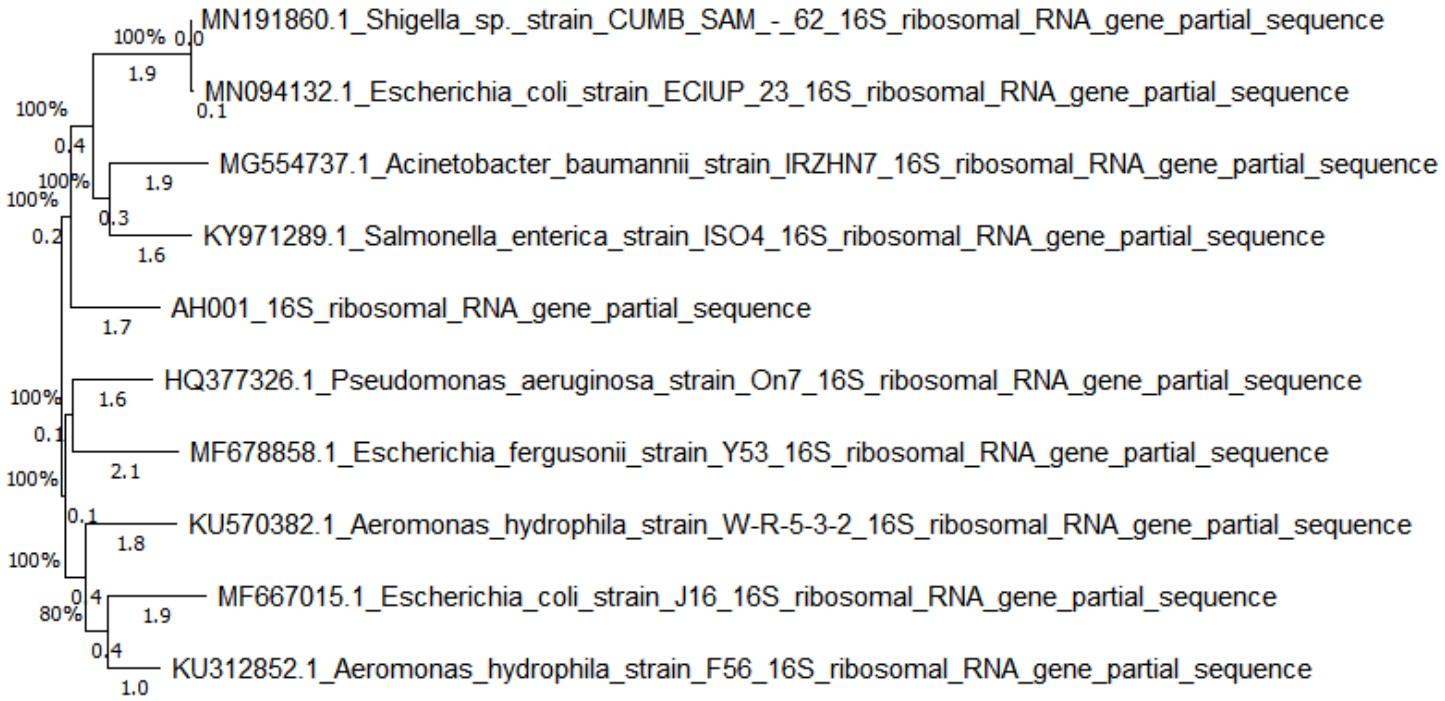

Figure 1

Evolutionary relationship between 16S rDNA gene of AH001 and other adjacent strains (This figure was created by MEGAX)

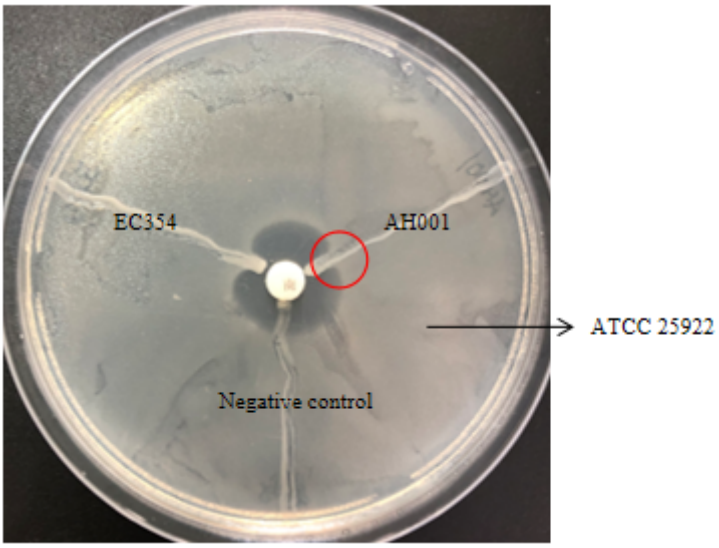

Figure 2

The result of Modified Hoge (EC354 is a isolate of E. coli isolated from sewage in the same batch as a positive control. AH001 and EC354 were tested by Modified Hoge test, and both isolates produced carbapenems. The principle is as shown in figure 1: carbapenems from AH001 and EC354 were inactivated and diffused into meropenem medium. There was not enough meropenem in this area to

inhibit E. coli ATCC25922, resulting in an apple-like indentation in the inhibition zone, which indicated that all the tested isolates produced carbapenems.