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RESEARCH ARTICLE



Molecular characteristics of carbapenem-resistant *Raoultella ornithinolytica*

XICAI SUN¹, YUGANG WANG², XUESONG WANG³ and
HONGGANG WANG^{4*} 

¹ Department of Archives, The First Affiliated Hospital of Weifang Medical University, Weifang, Shandong, China

² Department of Clinical Laboratory, Weifang Hanting District People's Hospital, Weifang, Shandong, China

³ Department of Clinical Laboratory, Weifang People's Hospital, Weifang, Shandong, China

⁴ Department of Clinical Laboratory, The First Affiliated Hospital of Weifang Medical University, Weifang, Shandong, China

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ABSTRACT

Aim of this study was to explore molecular characteristics and resistance mechanisms of carbapenem-resistant *Raoultella ornithinolytica* (CR-ROR) isolated from patients in a hospital in China. Three CR-ROR strains were collected and bacterial identification was done by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) Vitek-MS and by digital DDH analysis. VITEK 2 compact system and Kirby-Bauer (K-B) disk diffusion were used for antimicrobial susceptibility testing. Whole genome sequencing was carried out using the Illumina platform NovaSeq sequencer. ABRICATE software was used for the prediction of antibiotic resistance genes of three CR-ROR strains. The phylogenetic tree was constructed through genome SNPs to investigate the genetic relationship of three CR-ROR strains. Three CR-ROR (WF1357, WF2441, and WF3367) strains were collected in this study. Two strains were isolated from neurosurgery (WF1357 and WF2441), and one was isolated from pulmonology department (WF3367). All strains harboured multiple antibiotic resistance genes. Two strains (WF1357, WF2441) carried the *bla*_{NDM-1} gene, one of the strains (WF3367) carried the *bla*_{KPC-2} gene. Three CR-RORs were resistant to different antimicrobial agents including carbapenems. The three CR-ROR strains collected in this study and 51 CR-ROR strain genomes downloaded from NCBI, were divided into six evolutionary groups (A-F). In this study, three CR-ROR strains were found to have a higher level of resistance to antibacterial agents and carried multiple antibiotic resistance genes. The CR-ROR strains carrying multiple antibacterial resistant genes require the stringent monitoring to avoid the spread of multidrug-resistant bacterial strains.

KEYWORDS

Raoultella ornithinolytica, *bla*_{NDM-1}, *bla*_{KPC-2}, carbapenem resistance, Whole genome sequencing (WGS)

INTRODUCTION

Raoultella ornithinolytica is a Gram-negative bacterium belonging to the Enterobacteriaceae family which can cause hospital-acquired infections. In the 1980s, it was first classified as *Klebsiella*, but 16S rRNA gene and *rpoB* gene analysis showed inconsistent results, thereby it was reclassified in *Raoultella* genus in 2001 [1]. *Raoultella* can be isolated from water, soil, and plants. It is an opportunistic pathogen and can easily cause infections in people with low immunity or patients who underwent invasive surgeries. The increasing use of carbapenems in clinical practice enhanced emergence of carbapenem-resistant Enterobacteriaceae bacteria, that are now a great threat to patients under clinical therapy. Resistance to carbapenems is explained mainly by production of carbapenem-hydrolyzing enzymes. Carbapenemases

*Corresponding author.
E-mail: rmywf661188@wfmc.edu.cn

include enzymes of Class A, B, and D of Ambler's classification. The enzyme New Delhi metallo- β -lactamase-1 (NDM-1) belongs to Class B and the *bla*_{NDM-1} gene is mainly located on plasmids of varying sizes (*Klebsiella pneumoniae* 180 kb plasmid, *Escherichia coli* 140 kb plasmid) [2–3], suggesting that this gene can be transferred and transmitted among strains. A few bacterial *bla*_{NDM-1} genes have been reported to be located on the chromosomes [2–5]. At present, *bla*_{NDM-1}-carrying strains have been isolated mainly in Canada, the United Kingdom, Australia, Canada, Italy, South Africa, and others [2–6], and new cases of infection in these regions are on the rise.

K. pneumoniae carbapenemase (KPC) belongs to Class A carbapenemase as per the Ambler classification, and *bla*_{KPC-2} is one of the most common subtype of *bla*_{KPC}. Since the first isolation of KPC enzyme in *K. pneumoniae* in 2001, it has also been reported to exist in several bacterial species [7–10]. Zheng et al. [11] reported the possibility of widespread transmission of the *bla*_{KPC-2} gene in *Raoultella*.

In recent years, carbapenem resistance of Enterobacteriaceae has become a serious threat, and there have been increasing reports of carbapenem resistant *R. ornithinolytica* (CR-ROR). In this retrospective study, whole genome sequencing of three CR-ROR strains was carried out to understand the distribution and antibiotic resistance mechanism of CR-ROR in our hospital and to provide data support for the clinical treatment of infection caused by the CR-ROR strains.

MATERIALS AND METHODS

Collection of clinical isolates

CR-ROR strains were isolated from a tertiary teaching hospital with over 3,000 beds in northern China, between July 2017 and November 2020. Identification of bacterial strains was carried out using Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) Vitek-MS (Sysmex-bioMerieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Antimicrobials commonly used in the clinic include piperacillin-tazobactam (TZP), gentamicin (GEN), cefepime (FEP), imipenem (IPM), ceftriaxone (CRO), ampicillin (AMP), tobramycin (TOB), amikacin (AMK), cefotetan (CTT), ceftazidime (CAZ), ertapenem (ETP), levofloxacin (LVX), trimethoprim-sulfamethoxazole (SXT), ampicillin-sulbactam (SAM), ciprofloxacin (CIP) and aztreonam (ATM). The antimicrobial susceptibility testing was carried out using the VITEK 2 Compact (GN13) (Sysmex-bioMerieux, Marcy l'Etoile, France). The antimicrobial susceptibility testing for the drugs not included in the GN13 card like cefuroxime (CXM), meropenem (MEM), ceftazidime/sulbactam (CSL), cefotaxime (CTX), ceftiofur (FOX) and ceftazolin (CZO), was carried out using Kirby-Bauer (K-B) disk diffusion method (Oxoid, Hampshire, United Kingdom). The results of susceptibility testing were

interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (<https://clsi.org/>). *E. coli* ATCC 25922 was used as the quality control strain.

Bacterial genome extraction

Strains were streaked onto Columbia blood agar (AutoBio, Zhengzhou, China). Species identified by the Vitek-MS were inoculated overnight in Luria-Bertani (LB) broth (Oxoid, Hampshire, UK). Omega Bio-Tek Bacterial DNA Kit (Doraville, GA, USA) was used for the extraction of bacterial genomic DNA and the concentration and quality of the genomic DNA were checked using a NanoDrop spectrophotometer. The extracted DNA was stored at -80°C until further analysis.

Sequencing and sequence assembly

The whole genome of all the strains was sequenced using a paired-end library with an average insert size of 350 bp using a NovaSeq sequencer (San Diego, CA, USA). FASTQ files for each sample were Low-quality reads and adapters were trimmed by TrimGalore (v0.4.5, <https://github.com/FelixKrueger/TrimGalore>) and assembled independently using de novo assembler SPAdes 3.13.0 [12] with K-values of 21, 33, 55, 77, and 99 [13]. Additional assembly includes mapping contigs to reference plasmid sequences, checking overlapping paired ends and confirming the assembly by the PCR-based gap closure method.

Whole-genome sequencing (WGS) analysis

Antibiotic resistance genes were identified by NCBI database using Abricate 0.8 (<https://github.com/tseemann/abrigate>) [14]. MEGAX 10.1.8 was used to generate unrooted maximum-likelihood phylogenetic trees with a bootstrap iteration of 1,000 [15]. The data of bacterial strains for constructing the phylogenetic tree except for the three CR-ROR strains were obtained from the NCBI database. A total of 114 complete genomic data was obtained from the NCBI database (up to Sep 3, 2022), and 51 among them were identified to contain carbapenem resistance genes by Abricate. The 51 CR-ROR strains used in the phylogenetic tree were named by their accession numbers. The tree was generated based on single-nucleotide polymorphism (SNP) and was visualized by iTOL [16].

Nucleotide accession numbers

The sequences of the three CR-ROR strains were submitted to GenBank under BioProject PRJNA907537.

RESULTS

Characterization of *R. ornithinolytica*

Three CR-ROR strains were numbered WF1357, WF2441, and WF3367, all of which were strains obtained from elderly men with serious basic diseases (Fig. 1 and Table 1). Two strains were isolated from neurosurgery (WF1357 and WF2441), and one was isolated from pulmonology department (WF3367).



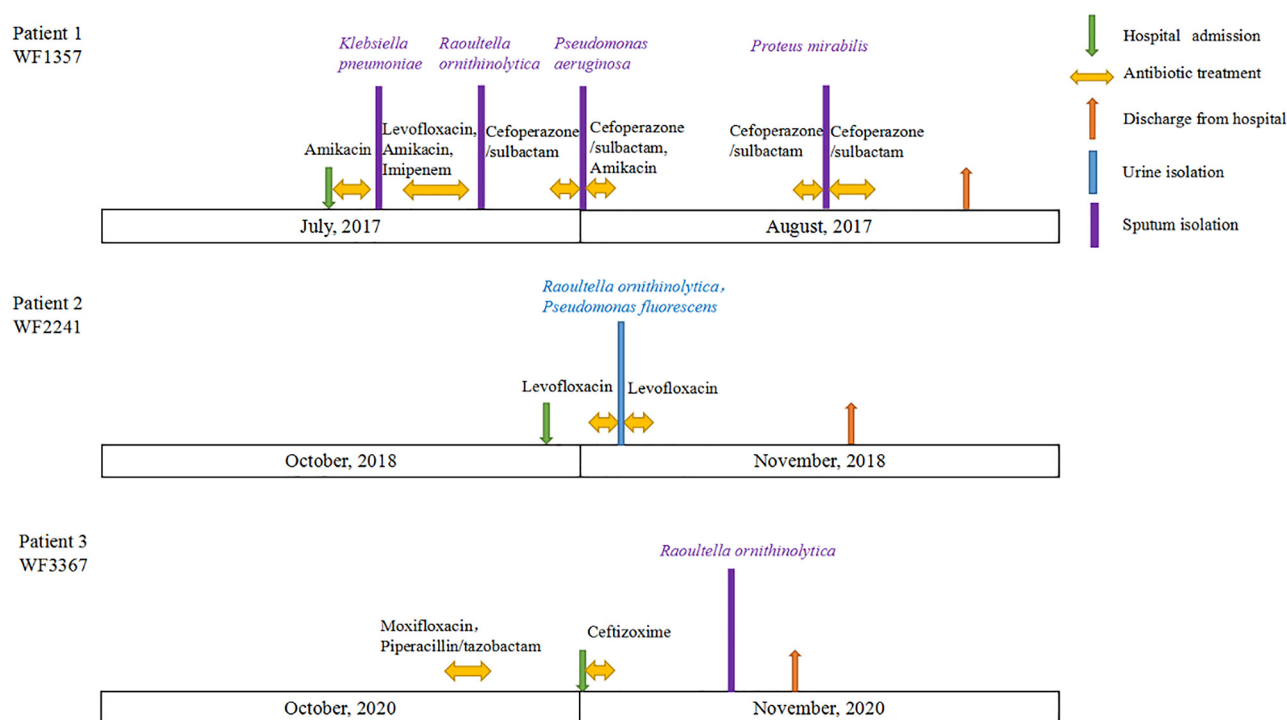


Fig. 1. Characteristics of *R. ornithinolytica* infections

Two strains were isolated from sputum samples (WF1357 and WF3367), and one strain was isolated from a urine sample (WF2441). WF1357 was obtained from the sputum samples of a 70-year-old male who was diagnosed with brainstem hemorrhage. Before the isolation of the CR-ROR strain, the patient had used antibacterial drugs like amikacin, levofloxacin, and imipenem. After CR-ROR isolation, cefoperazone/sulbactam sodium was used. The patient was in a coma after discharge, and given up treatment. WF2441 was obtained from urine samples of a 71-year-old male, who was diagnosed with intracranial space-occupying lesions. Levofloxacin was used before and after isolation. WF3367 was isolated from sputum samples of a 71 year-old-male, diagnosed with severe pneumonia. Before the isolation of the CR-ROR strain, levofloxacin, moxifloxacin, and piperacillin/tazobactam were used, but no antimicrobial were used after post isolation. The infection of WF2441 and WF3367 patients were controlled or resolved after therapy. Figure 1 depicts the specific antimicrobial treatment scheme.

Susceptibility results

Three CR-RORs were resistant to three carbapenems (IPM, MEM, ETP), and only sensitive to AMK. All three strains were resistant to more than three drugs and all belonged to multi-resistant bacteria. The specific drug sensitivity results of the three strains of bacteria are shown in Table 1.

Results of antimicrobial resistance gene detection

Through the prediction of the second-generation sequencing, 34 antimicrobial resistance genes were predicted for the

three strains, and two resistance genes *bla*_{NDM-1} (WF1357 and WF2441) and *bla*_{KPC-2} (WF3367) were predicted for carbapenem. WF2441 was found to contain 29 antimicrobial-resistant genes, WF1357 contained 21, and WF3367 contained 20. Three CR-ROR strains mainly carried some transfer-related pump proteins and outer membrane proteins. These genes can cause resistance to aminoglycoside, carbapenem, cephalosporin, fluoroquinolone, macrolide and peptides. WF2441 had more antimicrobial resistance genes than the other two isolated strains and mainly included genes for the resistance to sulfonamides, tetracyclines, aminoglycosides, and others (Fig. 2).

Phylogenetic analyses of *R. ornithinolytica*

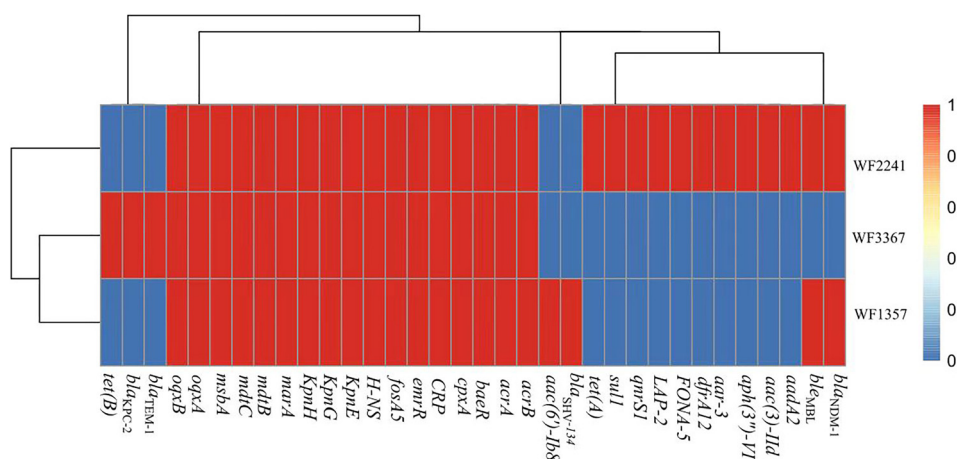
The three CR-ROR strains in this study and the 51 strains of *R. ornithinolytica* containing carbapenem resistance genes downloaded from NCBI were divided into six evolutionary groups (A-F) (Fig. 3). Three experimental strains in this study were distributed in groups A and F. Among them, WF1357 isolated in 2017 and WF2441 isolated in 2018 were both grouped in group A and they belonged to subgroups A1 and A2 respectively. WF3367 isolated in 2020 belonged to group F.

Compared with other countries, the three CR-ROR strains were closely related to strains from China (R2 red stripe). From the time point of view, the three strains closely resemble the strains isolated in 2014.

According to the characteristics of carbapenem-resistant genes of published sequences of *R. ornithinolytica*, CR-ROR strains carry several carbapenem-resistant genes, and five CR-ROR strains downloaded from NCBI carry multiple

	WF1357	WF2241	WF3367
Gender	Male	Male	Male
Age	70	71	71
Department	Neurosurgery	Neurosurgery	Respiratory
Diagnosis	Hemorrhage of brain stem	Intracranial space-occupying lesion	Broncho pneumonia
Specimen types	Sputum	Urine	Sputum
isolates date	28 Jul 2017	07 Nov 2018	10 Nov 2020
MIC ($\mu\text{g mL}^{-1}$)			
CRO	≥ 64	≥ 64	≥ 64
AMK	16	16	≤ 2
GEN	≥ 16	≥ 16	≤ 1
AMP	≥ 32	≥ 32	≥ 32
ATM	≥ 64	16	≥ 64
CAZ	≥ 64	≥ 64	≥ 64
CIP	≥ 4	1	≥ 4
LVX	≥ 8	1	≥ 8
FEP	≥ 64	≥ 64	8
IPM	≥ 16	8	≥ 16
ETP	≥ 8	≥ 8	≥ 8
SXT	≥ 16	≥ 16	≤ 1
TOB	≥ 16	2	8
CTT	≥ 64	≥ 64	≥ 64
TZP	≥ 128	≥ 128	≥ 128
SAM	≥ 32	≥ 32	≥ 32
K-B ($\mu\text{g mL}^{-1}$)			
MEM	6	12	14
CXM	6	6	6
CZO	6	6	6
CTX	6	6	10
CSL	8	6	10
FOX	6	6	6

Kirby-Bauer disk diffusion (K-B): meropenem (MEM), cefuroxime (CXM), ceftazolin (CZO), cefotaxime (CTX), ceftoperazone/sulbactam (CSL) and ceftoxitin (FOX).



carbapenem-resistant genes. In terms of time distribution, strains containing multiple genes were mainly isolated post 2018. In terms of geographical distribution, the CR-RORs were mainly distributed in Japan, the United States, and China.

In recent years, an increasing number of *R. ornithinolytica* have been isolated in clinical practice, and their drug



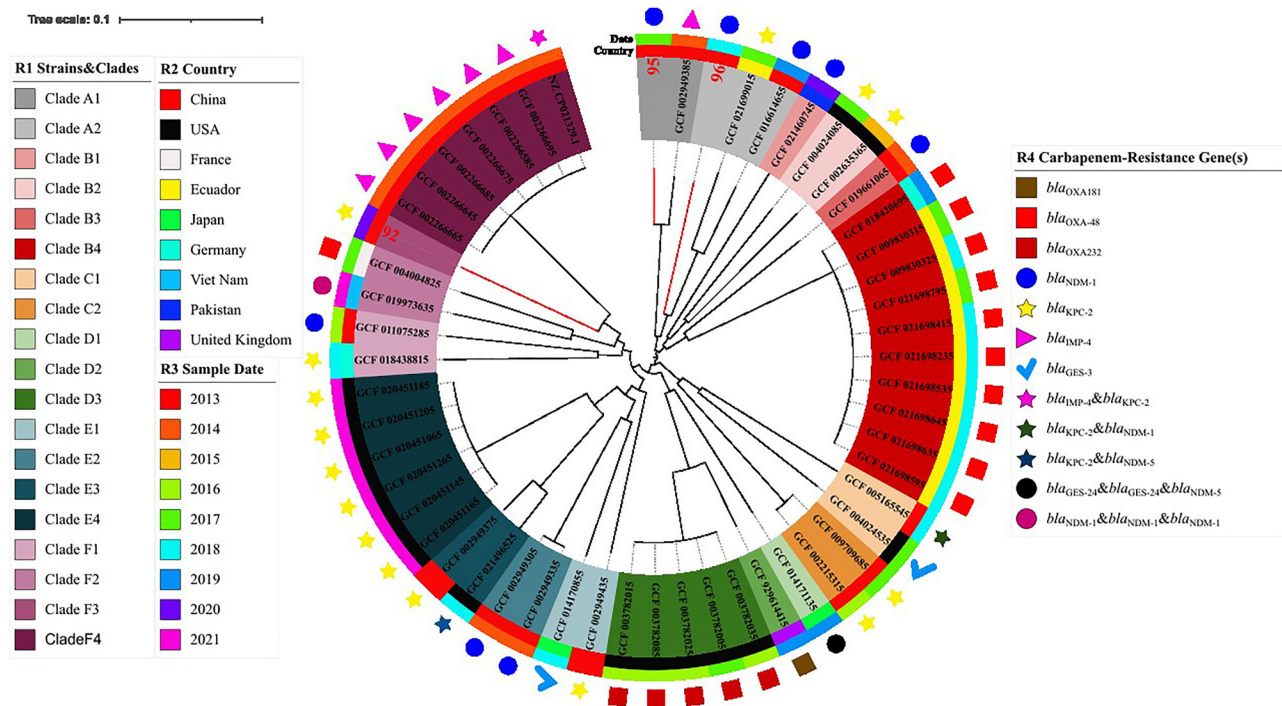


Fig. 3. Phylogenetic tree of CR-ROR strains (The red branches are strains in this study, and the other strains are the whole genome sequencing data with carbapenem resistance genes downloaded from the NCBI database)

resistance ability has been increasing with the extensive use of antibacterial drugs. There have been continuous reports of infection cases caused by *R. ornithinolytica*. However, there are only a few global studies on CR-ROR strains. This study involved the collection of three CR-ROR strains from a tertiary teaching hospital for whole genome sequencing and a retrospective study on the resistance gene, and case characteristics was carried out.

The three patients in this study suffered basic diseases and were infected with CR-ROR after admission, indicating that *R. ornithinolytica* is an opportunistic pathogen and can easily cause infections in people when their immune function is low. The three CR-ROR strains were isolated from hospitalized patients and all of them received broad-spectrum antimicrobial treatment before isolation. Two patients in this study (WF2441 and WF3367) were discharged after their recovery from relatively mild basic diseases and the treatment was given up in one patient (WF1357) due to the treatment failure of the relatively serious basic illness. The treatment failure of patients with relatively severe basic diseases indicates a poor prognosis of patients with serious illness, which is consistent with the results of Castanheira et al. [17] in which three patients with severe basic diseases died of CR-ROR infection. Therefore, it is speculated that the prognosis of patients with relatively mild underlying diseases and a normal immune system is generally good, while patients with severe underlying diseases may have a poor prognosis due to impaired immune capacity.

Due to the antimicrobial susceptibility testing results need several days, the treatment with clinical antimicrobial

mostly involves an empirical application, and no special antibacterial drugs such as carbapenems or polymyxins were used in the treatment plan. The antimicrobial susceptibility testing results showed that although the three CR-ROR strains were only sensitive to a few antimicrobial, all the strains were resistant to β -lactam antimicrobial which is consistent with the results reported by Zou et al. [18]. The symptoms of infection in two patients improved significantly after clinical empirical treatment. This could be the bacteria were resistant in vitro, but they were still effective in vivo.

The three CR-ROR strains carried a variety of antibiotic resistance. The WF2441 strain carried more antimicrobial resistance genes than the other two strains, especially genes associated with the resistance to antimicrobials like sulfonamides, tetracyclines and aminoglycosides. The *bla_{NDM}* gene is usually carried by *E. coli*, *Enterobacter cloacae* and *K. pneumoniae*. This gene has also been reported in other species of enterobacteria, such as *Klebsiella oxytoca*, *Klebsiella ozaenae*, *Citrobacter freundii*, etc [19]. These NDM-producing bacteria have been shown to cause hospital and community-acquired infections [19]. Bacteria producing *bla_{NDM-1}* are frequently found in environmental settings, indicating the environmental origin of *bla_{NDM}* genes in human pathogens. The indiscriminate use of antimicrobial drugs in some countries has aided in the rapid spread of *bla_{NDM-1}*-positive bacteria, posing a serious threat to public health [20]. The first case of CR-ROR carrying *bla_{NDM-1}* was reported by Khajuria et al. [21] in patients with urethral injury. However, there are few reports about *bla_{NDM-1}*-

harboring CR-ROR strains [22]. The *bla*_{KPC-2} is mostly carried by *K. pneumoniae*, and in this experiment WF3367 was found to carry *bla*_{KPC-2}, as well as a variety of other drug-resistant genes and resistance to several antibiotics. In this study, the gene environment of carbapenem resistance genes was not analyzed, and there were only three strains, in future research, we will collect more CR-ROR strains and analyze the gene environment of carbapenem resistance genes.

From the perspective of the structural homology of the three experimental strains, two strains with similar time of isolation have close homology, while WF3367 isolated in 2020 has high homology with strains isolated in 2014 in China, indicating that strains with similar time of isolation may have similar homology. However, for those with a different year of isolation bearing structural homology, a certain clone might have spread to different regions of China after a long period of transmission, or the clone might have become prevalent again after sometime. Elucidation of specific epidemic mechanisms requires further experimental validations. From the strain data downloaded from the NCBI database, it can be seen that in recent years, there have been strains co-expressing multi-carbapenem resistance genes in Asian and American countries. This may be due to the high prevalence of strains in these regions and the increased opportunities for bacteria to obtain foreign genes. Due to the continuous upgrading of antibacterial drugs used in these regions, strains expressing multiple resistance genes have been under constant exposure to antibacterial drugs, aiding them in the development of these resistant genes.

CONCLUSION

Global studies have reported the increased occurrence of CR-ROR strains in recent years. These strains exhibit strong antimicrobial resistance as they carry several resistance genes. The hospitals should therefore pay more attention to strengthen the monitoring of antimicrobial resistance, to control strictly hospital infections, and to avoid outbreak of CR-RORs in the hospital.

Authors' Contributions: H.W. designed the study. X.S., Y.W., and X.W. conducted the study, collected the data, and prepared the article. H.W. provided valuable advice and edited the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest: The authors have no conflicts of interest to declare.

Ethics: Given that all strains of this experiment were bacteria isolated from routine samples of the patients, and this study did not involve the patient's private information or data on animal subjects, this study was exempted from the requirement of ethical approval by the Ethics Committee of Weifang People's Hospital.

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