Characterization of carbapenem non-susceptible Gram-negative Bacilli isolated from the feces of 10,000 inpatients in Southern China

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Research

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Abstract

Background: Carbapenem non-susceptible Gram-negative bacilli (CNS-GNB) were dominant pathogen causing clinical infections. The human intestine was important reservoir of gram-negative bacilli (GNB), but there were few studies to analysis the prevalence of fecal colonization with them.

Methods: Fecal samples were collected from hosiptal screening test for GNB was conducted by using home-made MacConkey agar. Antimicrobial susceptibility was determined by the automatic microbiology analyzer and drug-resistant genes were characterized by polymerase chain reaction assays and DNA sequencing. The whole genome sequencing were used to analysis the characteristic of genetic structure of the isolates.

Results: A total of 680 CNS-GNB were collected. *Acinetobacter spp*. were the dominant genus (33.8%, 230/680) of the 22 genera. Carbapenemase genes were identified in 307 isolates (45.1%, 307/680), including 206 (30.3%, 206/680) bla_{NDM}; 51 (7.5%, 51/680) bla_{VIM-2}, 48 (7.1%, 48/680) bla_{IMP}, and seven (1.0%, 7/680) bla_{KPC-2}. The bla_{NDM} genes were first detected in three isolates, *Providencia vermicola*, *Achromobacter spp*., and *Cupriavidus gilardii*. Co-existence of bla_{VIM} and bla_{IMP} genes was detected in five isolates; *Achromobacter* co-producing VIM and IMP has not been previously reported. The *mcr-1* gene was identified in five strains of *Acinetobacter* and one strain of *Klebsiella pneumoniae*. In addition, we detected seven isolates harboring the bla_{AFM-1} gene, a novel metallo-β-lactamase gene. This was first genomic analysis of ST11 *Klebsiella pneumoniae* co-producing NDM-5 and mcr-1, which revealed that blaNDM-5 and mcr-1 are located on two different plasmids. The plasmid harboring blaNDM-5, which was composed of a typical IncX3-type backbone, and the *mcr-1* gene, was located between an IS30-like element ISApl1 and a PAP2-like encoding gene in the IncHI2-type plasmid.

Conclusions: the overall prevalence of fecal carriage of CNS-GNB in 10,000 stool samples was 7.45% (745/10000), and CNS-GNB producing carbapenemase were up to 45.1% (307/680). Most CNS-GNB cases were associated with infectious disease, multiple hospitalizations, or long-term care, and a high prevalence of underlying disease.

Background

Gram-negative bacilli are implicated in a wide range of diseases, such as pneumonia, meningitis, septicemia, and urinary tract infections [1]. According to China Antimicrobial Resistance Surveillance System (CARSS) monitoring data (http://www.carss.cn/), it has been shown that GNB accounted for 70.6% of the total number of bacteria which collected from 1,425 hospitals, and the strains were all isolated from clinical infection samples, mainly included sputum specimens (41.5%), urine specimens (18.8%) and blood specimens (9.2%). which are resistant to most available antibiotics and have developed built-in abilities to acquire new ways of resistance [2]. Carbapenem antibiotics have been reserved as drugs of last resort for salvage treatment of infections caused by multidrug-resistant gram-negative bacteria, but the emergence of CNS-GNB poses a global healthcare challenge because therapeutic options are limited. CNS-GNB represent difficult-to-treat infections in hospitalized patients and are associated with high mortality [3]. In recent years, the non-susceptible to carbapenem of GNB has spread rapidly, which has led to increased CNS-GNB, and asymptomatically colonized patients might act as important reservoirs for transmission.

The main non-susceptible to carbapenem mechanism of GNB is the production of carbapenemases capable of hydrolyzing carbapenems; these enzymes have been characterized into various classes, including Ambler class A β-lactamases, such as KPC and GES; Ambler class B metallo-β-lactamases, such as IMP, VIM, and NDM [4,5]; and Ambler class D oxacillinases (OXAs). Carbapenemase genes are generally located on plasmids, which can disseminate resistance horizontally by mobile genetic elements or mobile plasmids.

Colistin has been used as an effective clinical therapeutic against carbapenem non-susceptible bacteria. However, colistin resistance poses a substantial public health risk because it further limits treatment options in patients with infections caused by multidrug-resistant gram-negative bacteria, especially CNS-GNB. The recent discovery of transferable plasmid-mediated colistin resistance genes between bacteria has further increased the risk of spread of colistin resistance [6]. The *mcr* gene has been linked to colistin resistance and can be transferred via plasmids [7]. Strains that are not sensitive to carbapenems and show resistance to colistin can significantly increase the risk of death from clinical infection, so it is necessary to detect the presence of colistin resistance genes.

Few studies have described the prevalence of fecal colonization with CNS-GNB in China. In the present study, we collected 680 CNS-GNB isolated from fecal survey samples of 10,000 patients and studied the drug-resistant and transmission mechanism by analyzing resistance genes and the genomic structure.

Materials And Methods

Patients and specimens

All samples were obtained randomly and were not selected for on the basis of suspected enteric infection or diarrhea. A total of 10,000 fecal survey samples from in-patients who underwent routine stool examinations on the first day of hospitalization were prospectively and consecutively collected from July 2013 to June 2015 at Nanfang Hospital, a large, tertiary-level teaching hospital with 2,200 beds in Guangzhou, China.

Clinical data collection

The electronic medical records system were reviewed systematically for the clinical characteristics of the patients carrying fecal CNS-GNB. The following information was included: demographics (age and sex); geographical distribution of patients; colonization/infection status, type of infection, therapy received

and clinical outcome; Data on transplantation and immunosuppression, prior receipt of glucocorticoid, prior exposure to carbapenems, prior hospitalization, and invasive procedures such as surgery, use of mechanical ventilation, use of peripherally inserted central catheter were also recorded.

Bacterial Isolates

To screen for CNS-GNB isolates, approximately 0.5 mL or 0.5 g of stool sample was suspended in 0.5 mL of 0.9% sterile saline, and 10 μL of the resulting suspension was inoculated onto MacConkey agar medium (Beijing Land Bridge Technology, Beijing, China) containing 2 mg/L meropenem (Dainippon Sumitomo Pharma, Osaka, Japan) at 37°C for 18–24 h. Colonies surviving on MacConkey agar medium were stored at −80°C in nutrient broth containing 30% (v/v) glycerol. The study was approved by the Medical Ethics Committee of Nanfang Hospital Southern Medical University and conducted in compliance with the Declaration of Helsinki.

Species identification and anti-microbial susceptibility testing

Species identification and anti-microbial susceptibility testing was conducted with the BD Phoenix 100 Automated Microbiology System (Becton Dickinson and Co., Franklin Lakes, NJ, USA), and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines categories and minimum inhibitory concentration (MIC) breakpoints. Amplification and sequencing of a ~996 base pair fragment from the partial 16S ribosomal RNA (rRNA) gene sequence were performed to confirm the genus or species level of the low confidence (< 90 value) value isolates. Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI) sequence database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to identify related 16S rRNA sequences. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as control strains.

Investigation of carbapenemase genes and the colistin resistance genes

Detection of carbapenemase genes (bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GES}, bla_{GIM}, bla_{SIM}, bla_{OXA}, bla_{AFM}) and the colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*) were performed with polymerase chain reaction (PCR) followed by sequencing. Total DNA was amplified with the primers shown in Supplementary Table 1. Positive PCR amplifications were sequenced at the Beijing Genomics Institute (Shenzhen, China), and the sequences were compared with the NCBI database (www.ncbi.nlm.nih.gov).

Conjugation Assay

Conjugation experiments were performed in broth by using a sodium azide-resistant *E.coli* strain J53 as the recipient. The transconjugants were selected on Mueller-Hinton (MH) agar plates containing 4 μg/ml meropenem and 150 μg/ml sodium azide and MH agar plates supplemented with 4 μg/ml colistin and 150 μg/ml sodium azide. The MICs of imipenem, meropenem, ceftazidime, aztreonam and colistin against the donor, recipient and transconjugants were determined by using E-test strips (BioMérieux SA, La Balme-les-Grottes, France) obtained from Tian Kangxin (Beijing) Technology Co. Ltd. (Beijing, China) on MH agar plates.

Whole genome sequencing and analysis

It has been reported that the bla_{NDM} gene and *mcr* gene were mainly existed in *Escherichia coli* isolated from different sources, but the co-existence of bla_{NDM} gene and *mcr* gene in *K. pneumoniae* was rarely reported. In our study, one *K.pneumoniae* strain carrying *blaNDM-5* gene and *mcr-1* gene was detected in human feces, and which was resistant to carbapenem and colistin (Supplementary Table 2). Thus, the strain was selected for the whole genome sequencing. The whole genome was sequenced using the Single Molecule Real-Time (SMRT) sequencing platform with the PacBio sequencer and Illumina HiSeq at the Health Time Gene Institute (Shenzhen, China). The reads were denovo assembled using the HGAP (version 3.0). The prediction and annotation of the genome was achieved using GeneMarkS (version 4.6b, http://topaz.gatech.edu/GeneMark/) and BLAST (https://blast.ncbi.nlm.nih.gov/). The plasmids were typed using the PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/). The comparative and synteny analysis were generated using by BLAST and MUMmer (version 3.23, http://mummer.sourceforge.net/).

Results

Clinical data collection

Of 654 unique individuals with CNS-GNB samples, 549 (83.9%, 549/654) were from Southern China, mainly Guangdong province (97.3%, 534/549). The highest proportion was from Guangzhou in Guangdong (Supplementary Figure 2); 71 (10.9%, 71/654) were from Central China, 16 (2.4%, 16/654) from Eastern China; and 18 (2.8%, 18/654) from other regions in China. A total of 415 (63.5%, 415/654) were men and 239 (36.5%, 239/654) were women and the

median age was 55 years (range, < 1–91 years; Table 3). The samples were collected from almost all the wards across the hospitals, including ICU (34.3%, 224/654) and nearly 40 wards (65.7%, 430/654) of non-ICU departments. A total of 653 (99.8%, 653/654) had at least one underlying comorbid condition with a median Charlson comorbidity index of 2 (range, 0–4) and 1 (0.2%, 1/654) had no documented underlying condition. The most commonly reported conditions included diabetes (15.1%, 99/654) and neurological disorders (28.7%, 188/654); the ratio of malignancy (15.9%, 104/654) and chronic pulmonary disease (13.5%, 88/654) also surpassed other general underlying diseases. Bacterial infections were prominent, and most of the cases had a history of antibiotic use.

Identification of isolates

A total of 745 samples harbored CNS-GNB in 10,000 fecal samples, and 680 CNS-GNB were identified in 654 samples excluding 91 samples with *Stenotrophomonas maltophilia* only (Supplementary Figure 1). A total of 22 genera were identified, including nine types of 504 (74.1%, 504/680) carbapenem non-susceptible non-fermenting bacillus (CNS-NFB), eight types of 156 (22.9%, 156/680) carbapenem non-susceptible *Enterobacteria* (CNSE), and four types of 20 (2.9%, 20/680) other CNS-GNB. The genera isolated in the highest proportion were *Acinetobacter* spp. (33.8%, 230/680), *Pseudomonas* spp. (25.1%, 171/680), and *Enterobacter* spp. (9.0%, 61/680) (Table 1).

Antimicrobial susceptibility testing

Most of the isolates were resistant to cephalosporin, but they were sensitive to colistin. Amikacin resistance was identified in 147 isolates (21.6%, 147/680). Most of the CNS-GNB had a sensitivity rate of more than 80%, and sensitivity to amikacin of *Acinetobacter baumannii* was 54.8% (23/42). Most of CNSE strains had a resistance rate of less than 10% to amikacin; *E.coli* had a resistance rate of 25.9% (7/27). A total of 490 (72.1%, 490/680) isolates were ciprofloxacin resistant, 391 (57.5%, 391/680) isolates were levofloxacin resistant, and the susceptibility of different CNSE species to ciprofloxacin and levofloxacin varied. CNSE species were more sensitive to trimethoprim/sulfamethoxazole than non-fermenting bacillus, and 527 (77.5%, 527/680) isolates showed resistance to trimethoprim/sulfamethoxazole. A total of 354 (52.1%, 354/680) isolates were resistant to tetracycline, and non-fermenting bacillus species were more sensitive to tetracycline than CNSE (Table 2).

Investigation of carbapenemase and colistin resistance genes

A total of 307 (45.1%, 307/680) isolates harbored carbapenemase genes. Among them, bla_{NDM} was detected in 206 (67.1%, 206/307) isolates, including 188 (61.2%, 188/307) bla_{NDM-1}, 2 (0.7%, 2/307) bla_{NDM-4}, 15 (4.9%, 15/307) bla_{NDM-5}, and one (0.3%, 1/307) bla_{NDM-7}. In addition, 51 (16.6%, 51/307) isolates harbored the bla_{VIM-2} gene, and bla_{IMP} and bla_{KPC-2} were observed in 48 (15.6%, 48/307) and seven (2.3%, 7/307) isolates, respectively. Four isolates were found to co-exist with the bla_{IMP} and bla_{KPC} genes. Seven isolates were found to harbor the bla_{AFM-1} gene, a novel metallo-β-lactamase gene; these isolates include *Alcaligenes faecalis*, *Bordetella trematum*, *Comamonas testosterone*, and *Comamonas aquatica*. Five strains of *Acinetobacter* and one strain of *K. pneumoniae* were positive for the colistin-resistant gene *mcr-1* and co-existed with the bla_{NDM} gene. No other carbapenemase-related and colistin resistance genes were identified (Table 1). *Acinetobacter* was the most abundant carbapenemase-producing species (87.8%, 115/131), followed by *Escherichia coli* (70.4%, 19/27), *Providencia rettgeri* (64.7%, 11/17), *Citrobacter freundii* (64.3%, 9/14), and *Klebsiella pneumoniae* (58.5%, 24/41). *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* had the most carbapenemase gene types; bla_{NDM-1} was the most common carbapenemase gene in *Acinetobacter* spp. (100.0%, 115/115), bla_{NDM-5} was the most common type in *E. coli* (68.4%, 13/19), and bla_{KPC-2} was the most common in *K. pneumoniae*.

Conjugation

The transconjugants were selected on screening plates. PCR and sequencing by Sangon Biotech Co. Ltd. (Shanghai, China) revealed that the transconjugant NFYY0065-N contained the bla_{NDM-5} gene, and the transconjugant NFYY0065-M contained the *mcr-1* gene. Antimicrobial susceptibility testing showed that NFYY0065-N was resistant to imipenem, meropenem, ceftazidime, and aztreonam, whereas NFYY0065-M was resistant to colistin (Table 4).

Genomic analysis of K. pneumoniae co-producing bla_{NDM-5} and mcr-1

The chromosome was 5,235,159 bp with 57.55% GC content; it harbored 5186 genes, including five drug resistance genes encoding a class A broad-spectrum beta-lactamase, multidrug efflux transporter transcriptional repressor, tetracycline repressor protein, multidrug efflux transporter, and fosfomycin resistance protein (Table 5).

pAN65-MCR was 276,370 bp, possessed a typical IncHI2-type backbone, and contained 319 predicted open reading frames. It harbored the *mcr-1* gene, which was located between the IS30-like element ISApl1 and a PAP2-like encoding gene (Figure 1). The plasmid co-carried the resistance gene *blaCTX-M-14*. BLAST analysis of the complete nucleotide sequence against the NCBI database indicated the highest similarity (95% query cover and 99% nucleotide identity) to plasmid pMCR_WCHEC050613 (CP019214.2), which was from *E. coli* isolated from sewage in Chengdou. The genetic structure of *mcr-1* in plasmid pMCR_WCHEC050613 was also the same as pAN65-MCR. BLAST homology analyses revealed that pAN65-MCR has 93% identity and 99% query coverage

with pHNSHP45 [6], which was isolated from pigs. Comparative analysis of the genetic structures of *mcr-1* in the reference plasmids pHN6DS2 and pMCR1-PA revealed there were identical to pAN65-1, and pHNSHP45 had lost the *PAP2* gene downstream of *mcr* (Figure 2).

The bla_{NDM-5}-bearing plasmid pAN65-NDM was 46,161 bp with a GC content of 46.65% and belonged to the IncX3 group (Figure 3). BLAST analysis revealed high similarity (100% query cover and 99% nucleotide identity) to previously reported bla_{NDM-5}-positive plasmids, including the bla_{NDM-5}-positive plasmids of various sources, such as MH234502 (human, China) [8], CP027204 (sewage, China) [8], CP029245 (laying hen, China) [8], CP028705 (vegetable, China) [8], KX507346 (swine, China) [8], and CP034744 (goose, China) [8]. Comparative analysis revealed that these plasmids are highly similar, possessing the same backbone, which includes IncX3 replication. The genetic structure of the bla_{NDM-5} gene was identical to the plasmids pNDM5_020038, pK516_NDM5, and tig00001251; the mobile genetic elements IS30 and IS5 were upstream of bla_{NDM-5} and the genes *bleMBL* and *trpF* were downstream (Figure 4).

Discussion

Intestinal flora forms a major reservoir of bacteria, including multidrug-resistant bacteria. In this study, the overall prevalence of fecal carriage of CNS-GNB in 10,000 stool samples was 7.45% (754/10000), and the rate of carrying CNSE was 1.6% (156/10000). Hunan Province [9] revealed a higher rate of fecal carriage of CRE (8.5%) in hospitalized patients than that reported in a previous study (6.6%) in Fujian, China [10]. In France, 2.4% of 1,135 patients hospitalized or consulting in hospitals were shown to harboured carbapenem-resistant gram-negative bacilli (CR-GNB) isolates in fecal samples, and and the prevalence of carrying CRE was 0.7% (8/1135) [11]. In another study in France, 1.1% of individuals exhibited fecal carriage of CPE [12]. In Korea, 79 (26.3%) out of 300 persons harboured CR-GNB isolates in cotton swabbed stool samples [13]. Worldwide, the reported rate of CRE fecal carriage in hospitalized patients has varied from 0.3 to 69.5% [14-17]. Compared with these studies, it may be mainly due to a large amount of samples in our study and the collected strains were CNS-GNB instead of CR-GNB. The fecal samples collected in the present study contained 5.0% (504/10000) CNS-NFB, and *Acinetobacter spp.* was the dominant species accounting for 2.3% (230/10000); this result was similar to our previous report of the positive rate of *Acinetobacter spp.* [18], but different from other reports that the *Acinetobacter* was less than *Enterobacteriaceae* isolated from stool samples [11, 13]. In addition, *K. pneumoniae was the main* fecal CRE species in the study, which was similar to the pervious reports [9-13]. In general, the abundance of *Enterobacteriaceae* in the normal intestine is greater than *Acinetobacter*, but the positive rate of carbapenem non-susceptible *Acinetobacter* in the intestine was higher than *Enterobacteriaceae* in our study. *Acinetobacter* is the main pathogenic bacteria that causes nosocomial infections and is widely distributed in the environment, which is significant for the spread of drug-resistant bacteria. We isolated 22 genera of CNS-GNB from stool samples, some of which did not cause clinical infections, suggesting the possibility that drug resistance had spread widely between different strains.

CNS-GNB isolates in the present study exhibited high resistance rates to β-lactam antimicrobial agents, including cephalosporins, carbapenems, and the monobactam aztreonam; however, most isolates were sensitive to colistin. The susceptibility of different CNSE species to amikacin varied; most of the isolates exhibited low resistance to amikacin (< 20.0%). Liu et al. [9] reported that resistance to amikacin against CRE species isolated from fecal samples was less than 25%, while Wang *et al.* [19] reported that 1801 clinical CRE isolates showed susceptibility to amikacin (54.5%). Carbapenem non-susceptible strains isolated from fecal survey samples were more sensitive to amikacin than clinical strains. Pritsch et al. [20] reported that two of three carbapenamase-producing *A. baumannii* isolates were untreatable with locally available antimicrobial agents and were susceptible to colistin and amikacin only. In some cases, Our datas provided an effective choice for clinical treatment of infections.

A total of 307 (45.1%, 307/680) isolates carried carbapenemase genes, higher than that reported in France (18.5%, 5/27) [11]. There were 85 carbapenemase-producing *Enterobacteriaceae* (CPE, 54.5%, 85/156) in our study, while CPE were detected in Hunan (90, 54/60) and Fujian (40%, 8/20) [9, 10]. A possible explanation for this was the geographic difference or the time variance between the studies. bla_{NDM} (67.1%, 206/307) was the predominant carbapenemase gene in the present study; the four main types included bla_{NDM-1}, bla_{NDM-4}, bla_{NDM-5}, and bla_{NDM-7}. The positive rate of the bla_{NDM-1} gene was higher than for any other variant, consistent with previous reports [21]. In the present study, bla_{NDM} was identified in different species, and the sequencing of four different species indicated that bla_{NDM} can be transmitted between different species through mobile genetic elements or removable plasmids, including Enterobacteriaceae and non-fermenting Bacillus. Notably, NDM was mainly present in *Acinetobacter*, and we detected only one type of carbapenemase NDM in *Acinetobacter*, indicating that NDM production is an important factor in the resistance of *Acinetobacter* to carbapenem. Other metal enzymes such as VIM and IMP were mainly present in *Pseudomonas* spp., and KPC was mainly present in *K. pneumoniae*. Apart from the NDM, KPC, VIM, and IMP carbapenemases, no other carbapenemase genes were found. OXA-48, a carbapenemase gene found in many European countries, is rare in China. Whether the results of the present study, and those of previous studies in which OXA-48 was not detected, are typical, will require further investigation [22, 23]. In addition, 373 carbapenemase gene-negative GNB isolates were identified. Other mechanisms of carbapenem resistance have been recognized, which include overexpression of Extended Spectrum Beta-Lactamases (ESBLs) or AmpC β-lactamase, decreased membrane permeability due to porin loss, and expression of efflux pumps [24, 25]. The complexity of carbapenem resistance mechanisms in GNB and characteristics of the human gastrointestinal tract make the dissemination and transmutation of resistance genes more complex and frequent.

The bla_{AFM} gene was discovered in our research. This gene is a novel metallo-β-lactamase gene; its expression product can hydrolyze carbapenemase. Therefore, strains carrying the bla_{AFM} gene can also develop resistance to carbapenem antibiotics. In the present study, the bla_{AFM} gene was also present in different strains of the human gastrointestinal tract. Although it was not detected in common isolates of Enterobacteriaceae, the bla_{AFM} gene is often located on a plasmid and can spread through mobile genetic elements.

Colistin is a last-resort antimicrobial for infections caused by multidrug resistant Gram-negative bacteria. In 680 CNS-GNB, six isolates were resistant to colistin, which indicated low prevalence of the *mcr-1* gene among carbapenemase-producing fecal survey isolates. The *mcr-1* gene is a transferable resistance determinant against colistin. We detected five strains of *Acinetobacter* and one strains of *K. pneumoniae* co-producing mcr-1 and NDM-5 in the present study. It has been reported that both bla_{NDM} and *mcr* genes are commonly present in *E. coli* isolated from different sources [26-29], but the co-existence of the bla_{NDM}

and *mcr* genes in *K. pneumoniae* has rarely been reported. In this study, the plasmid harboring the *mcr* gene in *K. pneumoniae* was similar to other IncHI2-type plasmids present in *E. coli*, and upstream of the *mcr* gene was the common ISApl1 transposon. NDM-5 has been reported in many other countries, including India [30], China [31], Italy [32], America [33], and Spain [34]; it has also been isolated from pigs [35, 36], dairy cows [37], and vegetables [38]. In the present study, the IncX3 plasmid harboring bla_{NDM-5} were highly similar to IncX3 plasmids from various sources, suggesting their ability to be an efficient vehicle for bla_{NDM-5} dissemination among humans, animals, food, and the environment via the human intestine. Strains can capture the plasmid harboring *mcr* or bla_{NDM} genes, which leads to the generation of pan-drug resistance, and the phenomenon of co-existence of *mcr* or bla_{NDM} genes has spread to other isolates, which poses a significant challenge in the treatment of clinical infections.

In the present study, individuals with CNSE were primarily from the pneumology department, hematology department, ICU, and rehabilitation department. There were some common feature of individuals with CNSE, most of them had infectious diseases, multiple hospital admissions and higher infectious risk, the history of using antibiotics. Therefore, the frequent use of antibiotics was closely related to multidrug resistant bacteria. However, some individuals with CNSE did not have underlying diseases, and there was no record of previous antibiotic use. In addition, these individuals were from different regions of China, indicating that CNSE has been widely present in the intestines of normal individuals. If individuals carrying resistance genes have infections, failure of antibiotic treatment, as well as an increase in the risk of transmission to other patients, is likely.

In conclusion, the emergence of CNS-GNB in fecal matter poses a major concern. Our data showed that the overall prevalence of fecal carriage of CNS-GNB in 10,000 stool samples was 7.45%, *Acinetobacter* was the dominant bacterial species detected in CNS-GNB, and most of the strains were sensitive to colistin. The presence of carbapenemase genes was the main mechanism of CNS-GNB resistance to carbapenem antibiotics, mainly encoding NDM. Notably, bla_{NDM} genes were widely distributed in various isolates. The bla_{NDM} genes were first detected in *Providencia vermicola*, *Achromobacter* spp., and *Cupriavidus gilardii*, and *Achromobacter* co-producing bla_{VIM} and bla_{IMP} genes has not been reported. Genomic sequencing and analysis of *K. pneumoniae* harboring bla_{NDM} and *mcr* revealed that these genes were located on two different plasmids, which were similar to previously reported plasmids, suggesting their ability to be efficient vehicles for bla_{NDM-5} dissemination among humans, animals, food, and the environment via the human intestine. Our results highlight the fact that enhanced surveillance and health policies for the detection and control of these pathogens are urgently needed to limit the emergence and spread of such an organism. As the final antibiotic for the treatment of CNS-GNB infection, the emergence of MCR-producting strains reminds us to strengthen the management of antibiotic use and encourages the implementation of human feces surveillance, as well as actions to prevent and control the spread of multidrug resistant bacteria, especially bacteria resistant to carbapenems and colistin.

Abbreviations

CNS-GNB Carbapenem non-susceptible Gram-negative bacilli

CNS-NFB Carbapenem non-susceptible non-fermenting bacillus

CR-GNB Carbapenem-resistant Gram-negative bacilli

CNSE Carbapenem non-susceptible *Enterobacteriaceae*

CRE Carbapenem-resistant *Enterobacteriaceae*

CPE Carbapenemase-producing *Enterobacteriaceae*

ESBLs Extended Spectrum Beta-Lactamases

Declarations

Ethics approval and consent to participate: Verbal informed consent was obtained from all participants. The samples used were the specimens remaining after the patient's clinical examination. The study was approved by the Medical Ethics Committee of Nanfang Hospital Southern Medical University and conducted in compliance with the Declaration of Helsinki (No. NFEC-2014-002).

Consent to publish: Not applicable.

Availability of data and materials: All data generated or analyzed in this study are included in this published article and its supplementary information files.

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Authors' contributions: Xiaonv Duan carried out the experiments, data organization and analysis, and contributed to writing the manuscript and annotating the results. Xiaonv Duan and Si Li collected all the isolates and clinical data. Yuan Peng and Yingcheng Qin participated in a subset of the experiments. Yongyu Rui contributed to the design of the study and assisted in drafting of the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1

The carbapenemase genes and colistin-resistant genes of 680 carbapenem-non-susceptible GNB

Class	Genus	Species	No. of isolates						
			Total (n = 680)	Carpenmase genes (n = 307)	NDM−1 (n = 188)	NDM−4 (n = 2)	NDM−5 (n = 15)	NDM−7 (n = 1)	KPC−2 (n = 7)
Carbapenem-resistant non-fermenting bacillus (n = 504)	*Acinetobacter* (n = 230)	*Acinetobacter baumannii*	42	9	9				
		Acinetobacter junii	25	6	6				
		Acinetobacter johnsonii	14	5	5				
		Acinetobacter calcoaceticus	15	2	2				
		Acinetobacter lwoffii/haemolyticus	3						
		Acinetobacter spp.*	131	115	115				
	Alcaligenes (n = 4)	*Alcaligenes faecalis*	3	1	1				
		Alcaligenes spp.*	1						
	Achromobacter (n = 22)	*Achromobacter* spp.	22	10	1				
	Burkholderia (n = 28)	*Burkholderia cepacia*	26	1	1				
		Burkholderia spp.*	2						
	Chryseobacterium (n = 4)	*Chryseobacterium indologenes*	1						
		Chryseobacterium spp.*	3						
	Comamonas (n = 31)	*Comamonas testosteroni*	11	2					
		Comamonas aquatica	5	1					
		Comamonas spp.*	15	3					
	Delftia (n = 6)	*Delftia acidovorans*	6						
	Pseudomonas (n = 171)	*Pseudomonas aeruginosa*	45	16	1				1
		Pseudomonas alcaliphila	1						
		Pseudomonas putida	102	41	4				
		Pseudomonas pseudoalcaligenes	2	2					
		Pseudomonas spp.*	21	6	1	1			
	Sphingomonas (n = 8)	*Sphingomonas paucimobilis*	7						
		Sphingobacterium spiritovorum	1						
Carbapenem-resistant enterobacteriaceae (n = 156)	*Bordetella* (n = 8)	*Bordetella* spp.*	7	1					
		Bordetella avium	1						
	Cedeces (n = 2)	*Cedeces* spp.	2						
	Citrobacter (n = 14)	*Citrobacter freundii*	14	9	9				
	Escherichia (n = 27)	*Escherichia coli*	27	19	4	1	13	1	
	Enterobacter (n = 34)	*Enterobacter aerogenes*	12	5					
		Enterobacter cloacae	21	11	2				
		Enterobacter ludwigii	1	1	1				
	Klebsiella	*Klebsiella pneumoniae*	41	24	12		2		6

	(n = 41)				
	Proteus (n = 7)	*Proteus mirabilis*	7	2	
	Providencia (n = 22)	*Providencia rettgeri*	17	11	11
		Providencia vermicola	5	2	2
	Serratia (n = 1)	*Serratia marcescens*	1		
Other gram-negative bacilli (n = 20)	*Aeromonas* (n = 11)	*Aeromonas sobria*	6		
		Aeromonas hydrophila	1		
		Aeromonas caviae	2	1	
		Aeromonas veronii	1		
		Aeromonas spp.*	1		
	Cupriavidus (n = 2)	*Cupriavidus gilardii*	1	1	1
		Cupriavidus pauculus	1	1	
	Empedobacter (n = 5)	*Empedobacter brevis*	5		
	Pasteurella (n = 2)	*Pasteurella pneumotropica*	2		

*species belong to the genus other than those listed

Table 2

a Susceptibility (SIR) pattern of carbapenem-resistant non-fermenting bacillus

	Susceptibility SIR (%)										
	Acinetobacter spp. (n = 131)			*Acinetobacter junii* (n = 25)			*Acinetobacter baumannii* (n = 42)			*Pseudomona putida* (n = 102)	
	S	I	R	S	I	R	S	I	R	S	I
Amikacin	84.0	9.2	6.9	80.0	0.0	20.0	54.8	12.9	32.3	92.2	3.9
Gentamicin	19.8	6.9	73.3	20.0	0.0	80.0	45.2	0.0	54.8	21.6	1.0
Imipenem	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	2.0	5.9
Meropenem	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	5.9	3.9
Colistin	96.2	-	3.8	100.0	-	0.0	100.0	-	0.0	-	-
Trimethoprim/sulfamethoxazole	23.7	-	75.6	48.0	-	52.0	61.3	-	38.7	15.7	-
Chloramphenicol	-	-	-	-	-	-	-	-	-	0.0	0.0
Ciprofloxacin	9.9	2.3	87.8	84.0	0.0	16.0	38.7	0.0	61.3	9.8	0.0
Levofloxacin	47.3	29.8	22.9	68.0	16.0	16.0	38.7	61.3	0.0	9.8	5.9
Tetracycline	34.4	38.2	28.2	68.0	16.0	16.0	80.6	19.4	0.0	20.6	11.8

Table 2

b Susceptibility (SIR) pattern of carbapenem-resistant enterobacteriaceae

	Susceptibility SIR (%)								
	Escherichia coli (n = 27)			*Enterobacter cloacae* (n = 21)			*Klebsiella pneumoniae* (n = 41)		
	S	I	R	S	I	R	S	I	R
Amikacin	74.1	0.0	25.9	85.7	4.8	9.5	95.1	0.0	4.9
Gentamicin	37.0	0.0	63.0	66.7	0.0	33.3	80.5	0.0	19.5
Imipenem	0.0	29.6	70.4	0.0	9.5	90.5	0.0	9.8	90.2
Meropenem	0.0	11.1	88.9	0.0	14.3	85.7	0.0	9.8	90.2
Colistin	100.0	-	0.0	100.0	-	0.0	97.6	-	2.4
Trimethoprim/sulfamethoxazole	44.4	-	55.6	76.2	-	23.8	73.2	-	26.8
Chloramphenicol	33.3	3.7	63.0	0.0	0.0	100.0	0.0	0.0	100.0
Ciprofloxacin	44.4	3.7	51.9	76.2	0.0	23.8	73.2	2.4	24.4
Levofloxacin	14.8	14.8	70.4	81.0	9.5	9.5	46.3	2.4	51.2
Tetracycline	3.7	0.0	96.3	57.1	0.0	42.9	9.8	4.9	85.4

Drug susceptibility according to Clinical and Laboratory Standards Institute (CLSI) M100-S29 criteria. '-', not applicable.

R, resistance; I, indeterminate; S, sensitive.

Table 3

Demographic and underlying condition of Individuals with carbapenem non-susceptible Gram-negative bacilli

	No./Total (%)
Demographic	
Age (years)	
Male	415/654 (63.5)
Female	239 /654(36.5)
Age group, y	
0–18	44(6.7)
19–44	157(24.0)
45–59	199(30.4)
60–79	217(33.2)
≧ 80	37(5.7)
Age, median (range)	55(< 1–91)
Underlying condition	
None	1/654(0.2)
Chronic pulmonary disease	88/654(13.5)
Chronic renal insufficiency	29/654(4.4)
Bacterial infections[a]	204/654(31.2)
Neurological disorder	188/654(28.7)
Hepatobiliary diseases	34/654(5.2)
Cirrhosis	1/654(0.2)
Viral hepatitis	60/654(9.2)
Myocardial infarction	12/654(1.8)
Connective tissue disease	9/654(1.4)
Hematological diseases	57/654(8.7)
Diabetes	99/654(15.1)
Any malignancy	104/654(15.9)
Transplant recipient	2/654(0.3)
Immunodeficiency related diseases	1/654(0.2)
Other	99/654(15.1)
Charlson Comorbidity Index, median(range)[b]	2(0–4)
Previous exposure to healthcare	
History of antibiotic use	144/654(22.0)
Immunosuppressive medications in past 30 days	33/654(5.0)
Hospitalized within one year(except this time)	296/654(45.3)
Radiotherapy or chemotherapy many times	62/654(9.5)
Surgery within one year	54/654(8.3)
Current maintenance dialysis	5/654(0.8)
Trachoestomy	2/654(0.3)

[a]In addition to gastrointestinal infectious diseases

[b]Score range is 0 to 37; the higher the number, the more serious the constellation of coexisting comorbidities.

Table 4

MICs of imipenem, meropenem, ceftazidime, aztreonam and colistin against the donor, recipient and transconjugants.

Strain(s)	Description	MIC (μg/ml)				
		imipenem	**meropenem**	**ceftazidime**	**aztreonam**	**colistin**
NFYY0065	donor	32	32	256	48	8
E.coli strain J53	recipient	0.25	0.064	0.125	0.064	0.25
NFYY0065-N	Transconjugant containing bla_{NDM-5}	8	8	128	32	0.5
NFYY0065-M	Transconjugant containing mcr−1	0.25	0.064	0.125	0.064	4

Table 5

The antibiotic-resistance genes located on chromosome and plasmids in Klebsiella pneumoniae strain NFYY0065.

Sequence(s)	Size (bp)	G + C (%)	Resistance gene(s)
Chromosome	5,235,159	57.55	bla_{SHV-1}, acrR, tetR, emrD, fosA
Plasmid pAN65−3	46,161	46.65	bla_{NDM-5}, bleMBL
Plasmid pAN65−1	276,370	46.31	mcr−1, fosA, $bla_{CTX-M-14}$, aac(3)-Iva, aph(4)-Ia, sul2, sul3, floR, aadA3, cmlA1, aadA, abeS, aph(3)-Ia

Figures

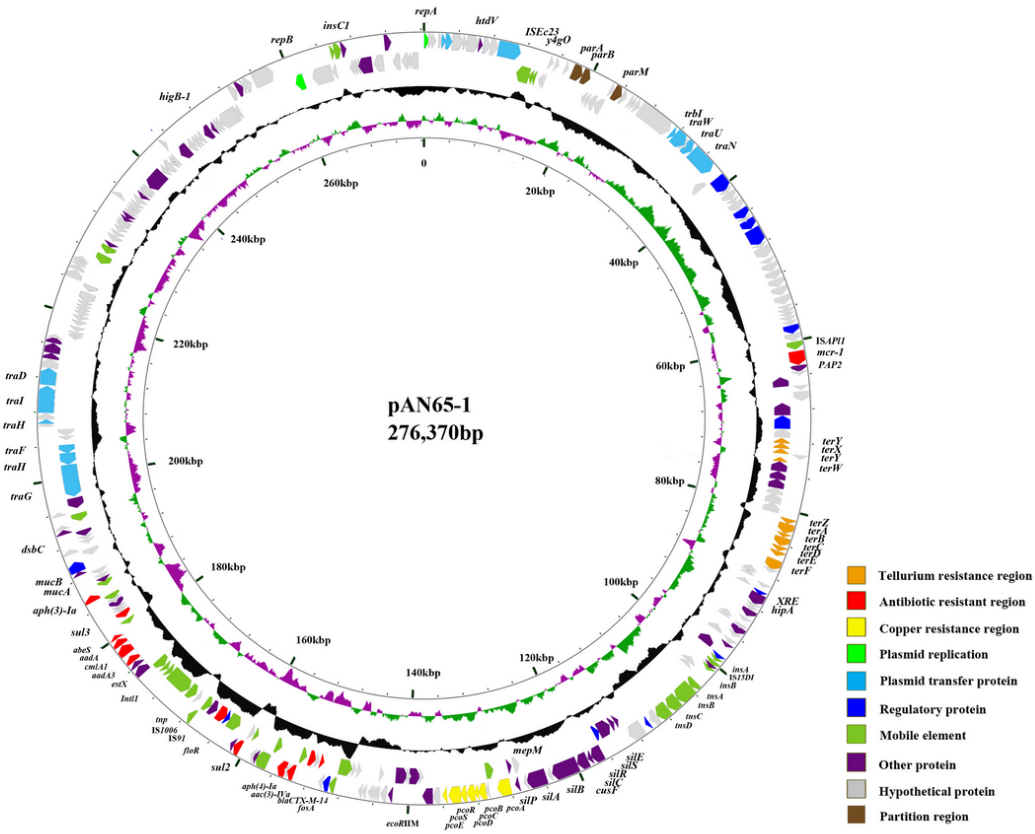

Figure 1

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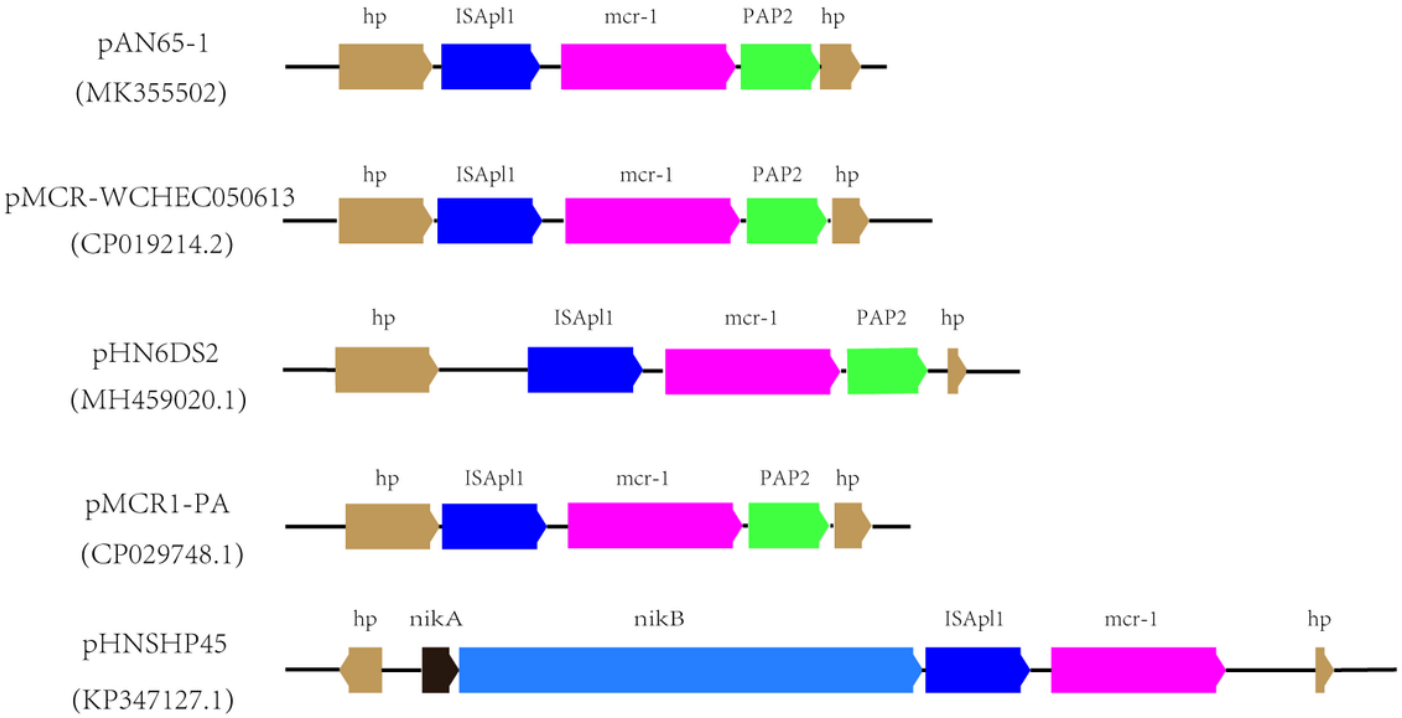

Figure 2

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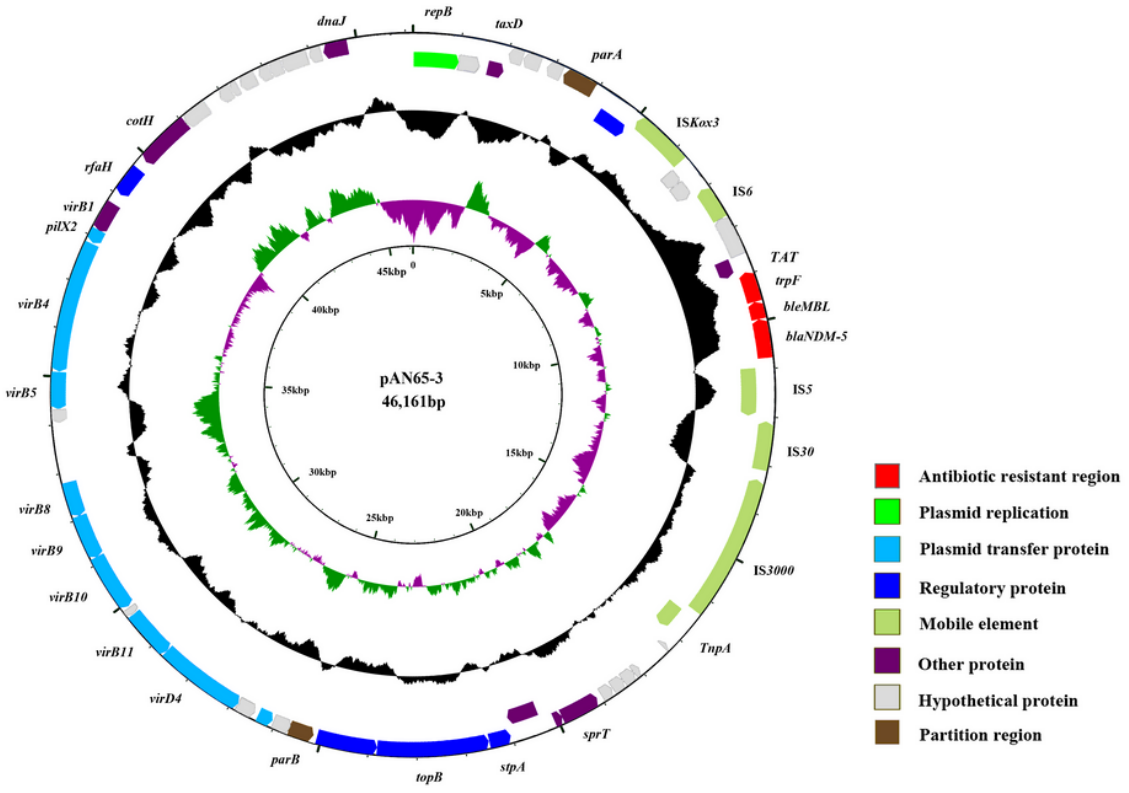

Figure 3

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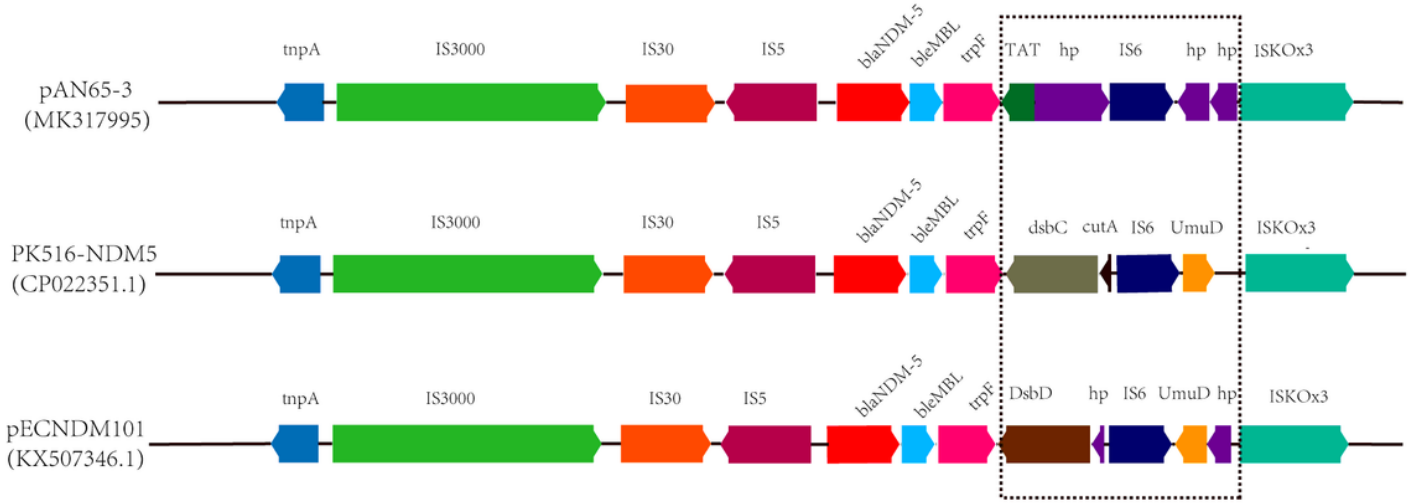

Figure 4

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