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ORIGINAL ARTICLE

Characterization of carbapenem-resistant *Klebsiella pneumoniae* in a tertiary hospital in Fuzhou, China

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Keywords
carbapenem resistance, *Klebsiella pneumoniae*, *KPC-2*, nosocomial infection, tigecycline.

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Abstract

Aims: The emergence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains has led to increased mortality and morbidity rates. Tigecycline, a new class of broad-spectrum glycyl-tetracycline antibiotics, has been used to target multi- and pan-drug-resistant bacterial infections. This study aimed to assess the molecular characteristics of CRKP in a tertiary hospital, and its susceptibility to tigecycline, to create a reference for hospital infection control and clinical drug use.

Methods and Results: We retrieved patient clinical information and CRKP characterization from medical records and detected the MIC of tigecycline using the micro-broth dilution method. Multi-locus sequence typing was performed, and antibiotic resistance genes associated with CRKP were detected by qPCR. A total of 166 CRKP strains were detected in the sputum, urine and blood among intensive care unit patients (average age, 69·6 years). The most infrequently observed resistance genes were amikacin resistance genes, followed by tobramycin resistance genes. *KPC-2*, *CTX-M9* and *CTX-M1* were the most frequently detected resistance genes.

Conclusions: No strain was resistant to tigecycline (MIC $\geq$ 8 μg ml^{-1}). Twenty-four sequence types were identified, with ST11 being the most common type.

Significance and Impact of the Study: Clinicians and infection control experts should be aware of CRKP prevalence to facilitate clinical treatment and improve nosocomial infection control.

Introduction

In recent years, *Klebsiella pneumoniae* drug resistance, pressured by antibiotic treatment, has been continuously increasing. Carbapenem antibiotics were effective against multi-drug-resistant and extended-spectrum beta-lactamase-producing strains; however, with the emergence of carbapenem-resistant bacteria, clinical treatment faces a new dilemma. In 2017, both Chinese and European antibiotic resistance monitoring networks showed an increase in the detection rate of carbapenem-resistant *K. pneumoniae* (CRKP). Specifically, an increase from 4·9% in 2013 to 9·0% in 2017 was reported in China, with an alarming increase of 26·9% in Shanghai. Meanwhile, the rate of European CRKP remained relatively unchanged, from 7·3% in 2014 to 7·2% in 2017, with the greatest change being from 62·3 to 64·7% in Greece (Simonsen 2018). According to the 2018 CHINET Resistance Monitoring Network, *K. pneumoniae* resistance to imipenem has risen rapidly from 16·1% in 2016 to 20·9% in 2017 and 26·1% in 2018. Therefore, CRKP resistance in China is increasing at an alarming rate, which has led to increased attention being paid to the CRKP drug resistance mechanism. Presently, the focus is on carbapenemase, the efflux pump, outer membrane pore proteins and the formation of biofilms.

However, the abundance of CRKP, its sensitivity to drugs and the percentage of strains carrying drug-resistant genes in our hospital remain unknown. Moreover, multi-locus sequence typing (MLST) and phylogenetic analysis have not been performed at our hospital. Therefore, the results of our research are expected to provide data that may be of clinical use in the treatment and control of these nosocomial infections.

Materials and methods

Patient characteristics and CRKP characterization

The clinical characteristics of the patients, including age, sex, inpatient ward and disease, were obtained from digitally stored medical records. *Klebsiella pneumoniae* strains were identified using the Vitek 2 system (BioMérieux, Marcy-l'Étoile, France) between January 2013 and December 2017. Antibiotic susceptibility tests were performed using a Vitek-2 Compact instrument. The ethics committee of Fujian Provincial Hospital (approval number: K2018-01-001) approved this study.

Susceptibility to tigecycline

Susceptibility to tigecycline was determined using the micro-broth dilution method. Tigecycline was purchased from Selleck Chemicals (Houston, TX; Lot: S140303) and was used at a concentration ranging from 0·06 to 128 μg ml^{-1}. Cation-adjusted Mueller–Hinton broth was purchased from Bio-Kont Co., Ltd (Wenzhou, China; Lot: HB6231-1). The ratio of the strain to the cation-adjusted Mueller–Hinton broth was 1 : 200, with 100 μl added to each well of a drug sensitivity plate. The plate was placed in an incubator with 5% carbon dioxide at 35°C overnight, and the results were observed on the second day.

Multi-locus sequence typing

Bacterial DNA was extracted using the Bacteria Genomic DNA Kit (CWBio Co., Beijing, China). MLST for *K. pneumoniae* was performed following methods previously described (Diancourt *et al.* 2005). The allelic profiles and the sequence types (STs) of each strain were determined using online databases (https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef).

Detection of antibiotic resistance genes

Primers for the detection of resistance genes (*CTX-M1*, *CTX-M9*, *KPC-2*, *Ompk35*, *Ompk36*, *Ompk37*, *IPM-4*, *NDM-1* and *OXA-48*) were designed using Primer Premier 5 (ver. 5.00) software and are presented in Table 1. Bacterial DNA was obtained as described above. qPCR was performed with an UltraSYBR mixture kit (CWBIO Co., Ltd) on a Cobas z 480 analyzer (LightCycler 480; Roche, Basel, Switzerland) with an initial incubation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Melting curve fluorescence was evaluated five times per degree Celsius from 60 to 95°C. Each reaction was carried out in triplicate.

Results

Demographic and clinical characteristics

The demographic and clinical characteristics of patients and specimens are shown in Table 2. The patients' ages ranged from 1 to 98 years with an average age of 69·57 ± 17·89, and the proportion of patients older than 60 years of age was 77·10%. Moreover, the number of male patients was 2·77 times that of female patients. The primary sources of specimens were sputum, blood, urine, alveolar lavage fluid, secretions, pus, pleural effusion, ascites and bile. The three most significant sources were sputum, which accounted for 42·17% of the specimens; urine, accounting for 27·10%; and blood, accounting for 15·67%. The diseases identified in our study primarily included pulmonary infection, cerebral infarction, gastrointestinal bleeding, nephrotic syndrome, bile duct stones, urinary tract infection and bloodstream infection.

Table 1 Primers of resistance genes associated with carbapenem-resistant *Klebsiella pneumoniae*

Genes	Primer sequence (5′→3′)	Product length (bp)	Annealing temperature (°C)
CTX-M1	AGGAAGTGTGCCGCTGTAT AGATTCGGTTCGCTTTCAC	216	55
CTX-M9	ACGCAGGTGCTTTATCGC TGTGCCGTTGACGTGTTTT	183	57
IMP-4	GCAGAGCCTTTGCCAGATT CGTGGGGATGGATTGAGA	288	57
KPC-2	GCGGCTCCATCGGTGTGTA TGGCGGCGGCGTTATCA	282	60
NDM-1	ATGTCTGGCAGCACACTTCC CCGCAACCATCCCCTCT	300	58
OmpK35	CAGGTCCTTGCCTTTGGTCT CAACGGTATCGCACTGTCTG	283	58
OmpK36	GCAAAGCCCAGGGAACC CGTACCGCCTTGAAACAGA	254	57
OmpK37	GGCGATTACGGCTCCTT TGCTGCGGTTATTGGTG	265	55
OXA-48	CCATAAGGCAACCACCACA CCATAACCAACACGCTTCACT	183	57

Table 2 Demographic and clinical characteristics of patients and specimens

Characteristics (unit)	Numerical data, *n* (%)
Median age (years)	73
Interquartile range (years)	61–83
Category by age (years), *n* (%)	
<20	1 (0·6)
20–40	13 (7·8)
41–60	24 (14·5)
61–80	74 (44·6)
81–100	54 (32·5)
Gender, no. males (%)	121 (72·9)
Source, *n* (%)	
Sputum	70 (42·2)
Urine	45 (27·1)
Blood	26 (15·7)
Others	25 (15·0)
Comorbidities, *n* (%)	
Brain diseases	50 (30·1)
Pulmonary infections	35 (21·1)
Diseases of the digestive system	28 (16·9)
Diseases of the urinary system	10 (6·0)
Heart diseases	9 (5·4)
Others	34 (20·5)
Origin, *n* (%)	
Intensive care unit	105 (63·3)
Neurosurgery	13 (7·8)
Neurology	5 (3·0)
Senior officials' inpatient ward	5 (3·0)
Others	38 (22·89)
Average hospital stay (days)	50
Interquartile range (days)	22–58
Outcomes, *n* (%)	
Crude 7-day mortality	1 (0·6)
Crude 30-day mortality	5 (3·4)
Total mortality	8 (5·4)

The four most common diseases were brain diseases (30·12%), based on samples from neurology, neurological surgery and brain-related diseases in the intensive care unit (ICU); respiratory diseases (21·08%); digestive tract diseases (16·87%); and urinary system diseases (6·02%). Patients in the hospital were mainly admitted in the ICU, gastroenterology, neurology, neurosurgery, cardiovascular medicine, hepatobiliary surgery and senior officials' inpatient wards. The four most common wards were the ICU (63·25%), neurosurgery (7·83%), neurology (3·01%) and senior officials' inpatient wards (3·01%). The 7-day, 30-day and total mortality numbers of CRKP-infected patients were as follows: 1 (0·6%), 5 (3·4%) and 8 (5·4%) respectively.

Susceptibility of CRKP to antibiotics

The detection rate of CRKP strains collected in this study was 15·74% (166/1055). All strains showed multi-drug resistance. Resistance rates to amikacin, tobramycin, minocycline, gentamicin, sulfamethoxazole and cefoperazone/sulbactam were 41·60, 61·60, 64·00, 68·80, 69·20 and 69·60% respectively. Cefotetan, levofloxacin, ciprofloxacin, cefepime, ertapenem, aztreonam, piperacillin/tazobactam, ampicillin/sulbactam, ceftazidime and imipenem resistance rates were >90%. The resistance rate against IPM, according to the Clinical & Laboratory Standards Institute (CLSI) M100-S27 standard (IPM $\geq$4 μg ml^{-1} or inhibition zone diameter $\leq$19 mm), was 100%, as shown in Fig. 1. Tigecycline MIC values are shown in Fig. 2. MICs were mainly concentrated at 0·25 and 0·5 μg ml^{-1} (73·5%), and no tigecycline-resistant strains were found, according to the Food and Drug Administration standard (MIC $\geq$8 μg ml^{-1}).

Sequence alignment and phylogenetic analysis

We compared our sequence analysis to the MLST database and identified a total of 24 ST strains (ST1, ST11, ST15, ST23, ST34, ST36, ST37, ST147, ST231, ST313, ST369, ST550, ST571, ST859, ST873, ST1224, ST1517, ST1647, ST2230, ST2235, ST2370, ST2894, ST3034 and ST3087). These strains were divided into two distinct groups: group 1 contained 18 strains (ST1, ST11, ST15, ST23, ST34, ST36, ST37, ST147, ST231, ST313, ST550, ST859, ST873, ST1517, ST2370, ST2894, ST3034 and ST2230), as shown in the phylogenetic analysis in Fig. 3; whereas group 2 contained six strains (ST369, ST571, ST1224, ST1647, ST2235 and ST3087). The most abundant ST strains of carbapenem-resistant *K. pneumoniae* in our hospital were as follows: ST11, 80·12% (133/166); ST15, 3·01% (5/166); ST147, 2·41% (4/166); and ST23 1·81% (3/166). Moreover, ST258 was determined to be the network download strain (BAA1705), and ST37 was the outbreak strain in the neonatal ICU (NICU). (Chen *et al.* 2019).

CRKP resistance genes

The results of drug resistance gene analysis are shown in Fig. 4. *KPC-2* was identified in 75·90% (126/166), *CTX-M9* was identified in 68·07% (113/166), *CTX-M1* was identified in 7·83% (13/166), *OmpK35* deletion was identified in 4·82% (8/166), *OmpK36* deletion was identified in 93·98% (156/166) and *OmpK37* deletion was identified in 5·42% (9/166) of all strains. *IPM-4*, *NDM-1* and *OXA-48* were not detected.

Discussion

Klebsiella pneumoniae is the leading cause of nosocomial infections worldwide, and its incidence is on the rise

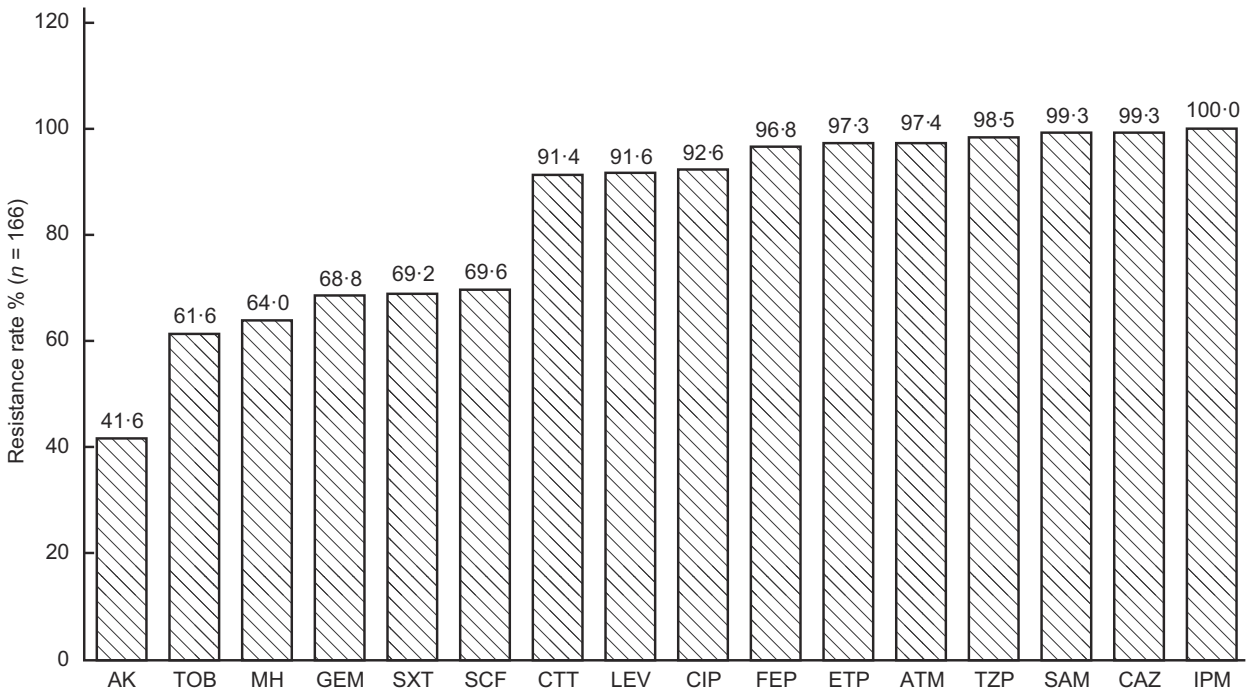

Figure 1 Susceptibility of CRKP to antibiotics.

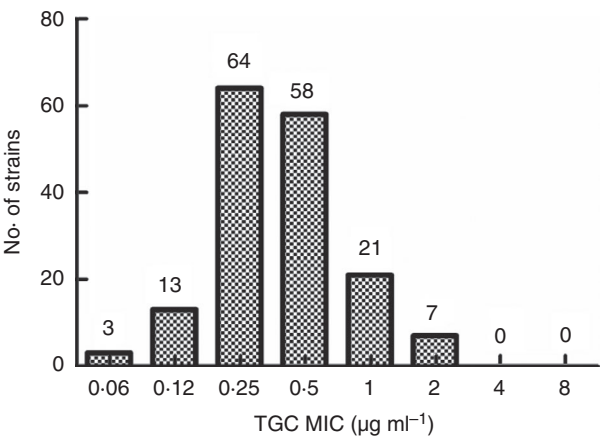

Figure 2 MICs of tigecycline against CRKP.

(Saidel-Odes and Borer 2013). Although its prevalence is second only to that of *Escherichia coli*, its drug resistance is broader and more problematic than that of *E. coli*. Treating CRKP is thus exceedingly difficult. Xu *et al.* (2017) retrieved reports published before December 22, 2015, that included terms such as '*Klebsiella pneumoniae*', 'drug resistance', and 'carbapenemase', 'imipenem', 'meropenem', or 'ertapenem'. Their systematic review and meta-analysis of the mortality rates of infected patients unveiled that the mortality rate for 2462 patients with CRKP was 42·14%, whereas that for patients with carbapenem-sensitive *K. pneumoniae* infection was 21·16%. Moreover, the mortality rates of patients with bloodstream or urinary tract infections were 54·30 and 13·52% respectively. The mortality rates of patients admitted to the ICU or receiving solid organ transplantation were 48·9 and 43·13% respectively. Geographical differences have also been observed: North American, South American, European and Asian studies have reported mortality rates of 33·24, 46·71, 50·06 and 44·82% respectively. Therefore, it is crucial to monitor drug sensitivity, as well as drug resistance genes and the molecular epidemiological characteristics of CRKP.

A total of 166 CRKP strains were collected in this study, primarily from patients over 60 years of age, which is consistent with previous reports (van Duin *et al.* 2014; Meng *et al.* 2019). Han *et al.* (2017) reported a CRKP prevalence of 24·6% (946/3846); among these, 507 (53·6%) were from respiratory specimens, 350 (37·0%) from the urinary system and 9 (9·4%) from blood, which is also consistent with our research. The detection rate of CRKP in our hospital was 15·74%, which was higher than the averages in the Fujian province (10·7%) and all of China in 2017 (9·0%). There were significant differences between regions in our study; Shanghai had the highest resistance rate (26·9%), whereas Qinghai had the lowest (0·3%). Our results were quite different from those of the sensitivity analysis that included 244 CRKP strains performed by Li *et al.* (2019), who concluded that, because

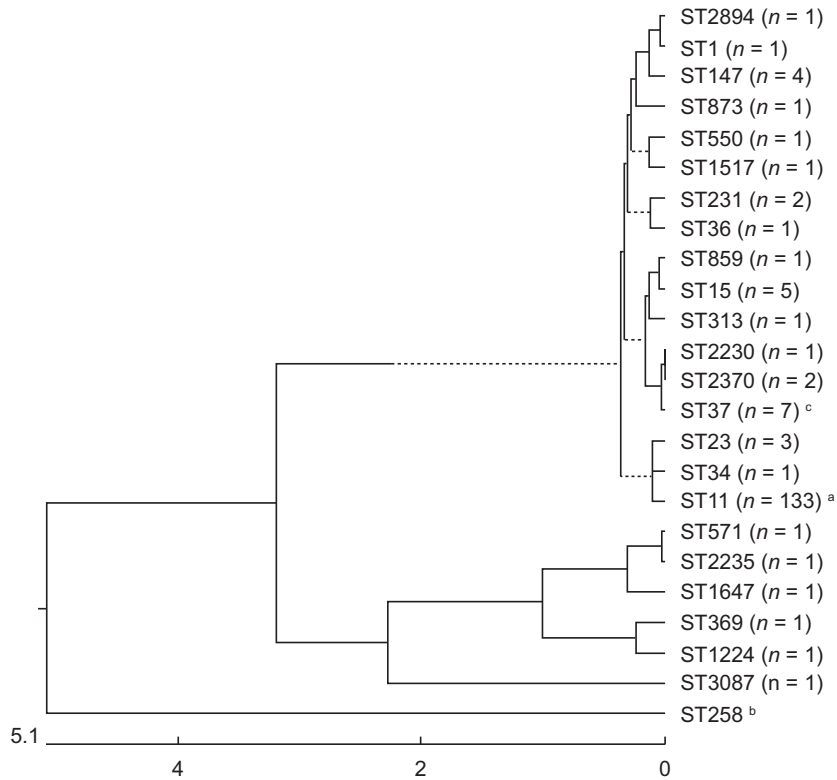

Figure 3 MLST and phylogenetic trees of CRKP. a, ST11 is the most common type; b, ST258 is the network download strain BAA1705; c, outbreak strains in the NICU.

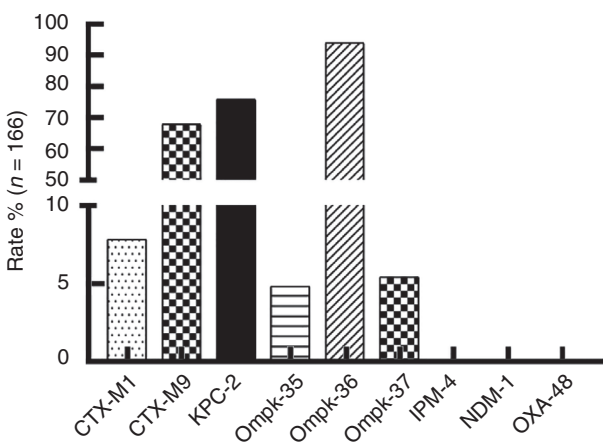

Figure 4 CRKP resistance genes.

of its high sensitivity rate (95·5%), tigecycline is still the best choice for CRKP. The rate of sensitivity to the remaining antibiotics was only slightly higher than 30%, except for fosfomycin, which had a sensitivity rate of 35·2%. The differences in sensitivity could be related to the different CRKP strains that are prevalent in each region. Tigecycline-resistant *K. pneumoniae* strains were not detected, which may be because the period of initial use of tigecycline is short, and it is a specific antibiotic in our hospital. In addition, to detect the MIC of tigecycline, the appropriate detection method should be selected. When the method shows mediation or drug resistance, the micro-broth dilution method or E-Test strip is required to determine the exact MIC.

Globally, the most common CRKP strains are ST11 and ST258; ST11 is the main ST in China (64·2%), and ST258 is the most common in the United States (70·0%) (Kitchel *et al.* 2009; Andrade *et al.* 2011; Qi *et al.* 2011). Previous studies (Chen *et al.* 2014) have shown that ST258 is a hybrid clone: 80% of the genome originates from an ST11-like strain, and 20% originates from an ST442-like strain. Lu *et al.* (2018) studied 174 CRKP strains from hospitalized patients at the Affiliated Hospital of Sun Yassin University Medical Sciences, and approximately 98·0% of CRKP strains belonged to ST11. The proportion of the ST11 strain in our hospital was similar to that described by Shu *et al.* (2019), who reported a proportion of 78·0%. MLST and evolutionary tree typing showed that the strains were mainly divided into two branches. No other ST has caused an epidemic, except for a NICU

outbreak of ST37 observed in our previous study (Chen *et al.* 2019).

In our hospital, the *KPC-2* gene was most commonly detected (75·9%), followed by the *CTX-M9* gene (68·07%). *OmpK36* deletion was observed at a rate of 93·98%; however, *IPM-4*, *NDM-1* and *OXA-48* were not detected. Galani *et al.* (2019) detected 300 CRKP strains in hospitals across Greece and found *KPC-2* (66·7%), *NDM* (16·7 %), *VIM* (7%) and *OXA-48* (4·0%); 14 strains carried both *KPC* and *VIM* (4·7%), two strains carried both *NDM* and *OXA* (0·7%) and one strain carried both *KPC* and *OXA* (0·3%) resistance genes. Tian *et al.* (2018) retrospectively analysed the drug-resistant phenotypes and clinical molecular epidemiology of 170 CRKP strains in Shanghai from January 2016 to December 2017 and found that the most abundant gene was $bla_{\text{OXA-232}}$ (42·35%), followed by $bla_{\text{NDM-1}}$ (20·59%), $bla_{\text{KPC-2}}$ (17·65%), $bla_{\text{NDM-5}}$ (16·47%) and $bla_{\text{IMP-4}}$ (1·18%). Furthermore, they found that the genes present were related to different ages and epidemic strains in different regions. The major drug resistance gene in China and the United States is *KPC*, found in >90% of strains, with *KPC-2* being the most common. The *NDM-1* carrier rate is <10% in China and <5% in America (Deleo *et al.* 2014; Li *et al.* 2014). We suggest that screening be performed to detect other drug resistance genes to better understand the epidemiological trends of drug-resistant strains and provide a theoretical basis for infection control and clinical treatment.

Here common drug resistance genes were detected. However, the expression of efflux pump genes and biofilm formation among the 166 CRKP strains were not analysed. The drug resistance gene carrier vehicles, such as plasmid vectors, insert sequences, integrins or other drug resistance gene mobile elements, were not assessed in this study. Therefore, the present investigation of CRKP strain carbapenem resistance mechanisms must be complemented by further studies. Future work will aim to develop clinical solutions to treat and prevent multi- and pan-drug-resistant bacterial infections.

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Conflict of Interest

No conflict of interest declared.

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