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ORIGINAL ARTICLE

Outbreak of NDM-1-producing *Klebsiella pneumoniae* ST76 and ST37 isolates in neonates

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Abstract The purpose of this study was to investigate the epidemiological characteristics of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) in Shanghai Children's Hospital in China. Twenty-two non-duplicate CRKP strains were collected from pediatric patients between March and June in 2014. Antimicrobial susceptibility testing was conducted by the agar dilution method. Beta-lactamases were characterized by polymerase chain reaction (PCR) and DNA sequencing. The transferability of $bla_{\text{NDM-1}}$ was investigated by conjugation experiment. The plasmids bearing antibiotic resistance genes were characterized by S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and Southern hybridization. Clonal relatedness was evaluated by PFGE and multilocus sequence typing (MLST). The clinical data of patients were retrospectively reviewed. The 22 CRKP strains were resistant to most of the antimicrobial agents tested, except tigecycline and colistin. Overall, 59, 77, and 100 % of these strains were resistant to imipenem, meropenem, and ertapenem, respectively. The $bla_{\text{NDM-1}}$ was positive in 77.3 % (17/22) of the CRKP strains, of which the 16 isolates from inpatients were designated as ST37 ($n=9$) and ST76 ($n=7$) and one isolate from an outpatient belonged to ST846. The 17 $bla_{\text{NDM-1}}$-positive isolates belonged to PFGE type A ($n=9$), type C ($n=7$), or type B ($n=1$). The plasmids bearing $bla_{\text{NDM-1}}$ could be transferred into recipient *Escherichia coli* J53 through conjugation in 88.2 % (15/17) of the strains. The hybridization results showed that the plasmids carrying the $bla_{\text{NDM-1}}$ gene were approximately 50–240 kb in size. This is the first report of an outbreak caused by NDM-1-producing *K. pneumoniae* ST76 and ST37 among neonates.

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has become a major public concern, which was mainly mediated by the production of carbapenemases [1]. As a novel carbapenemase, NDM-1 was initially reported in 2008 from the *K. pneumoniae* and *Escherichia coli* isolated from a Sweden patient who had received medical care in India [2]. Since then, carbapenem-resistant Enterobacteriaceae (CRE) bearing $bla_{\text{NDM-1}}$ and its nine minor variants have been identified all over the world [3–5].

Since the first NDM-1-producing *K. pneumoniae* was detected in Nanchang, China in 2013, they have spread rapidly in mainland China [6]. To date, NDM-1-producing *K. pneumoniae* have been reported in several regions of China [7, 8]. However, outbreak in neonates remains uncommon in China. In this study, we observed a soaring

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number of CRKP sourced from neonatal intensive care unit (NICU) and neonatal wards in less than three months.

Materials and methods

Bacterial strains

Twenty-two non-duplicate sequential strains of CRKP were isolated from Shanghai Children's Hospital to investigate the epidemiological characteristics. Most (86.5 %, 19/22) of the 22 CRKP were isolated from sputum, and 4.5, 4.5, and 4.5 % were isolated from trachea cannula, urine, and pus, respectively. All the strains were identified using the VITEK 2 Compact system (bioMérieux, France). *Escherichia coli* ATCC 25922, *Salmonella* ser. *Braenderup* H9812, and *E. coli* J53 (sodium azide resistant) were used as the quality control for antimicrobial susceptibility testing, reference marker for pulsed-field gel electrophoresis (PFGE), and recipient strain for conjugation experiment, respectively.

Antimicrobial susceptibility testing and β-lactamase characterization

Antimicrobial susceptibility testing was performed using the agar dilution method. The results were interpreted following the criteria of the Clinical and Laboratory Standards Institute (CLSI; 2014) [9]. Breakpoint minimum inhibitory concentrations (MICs) of tigecycline were determined following the guidelines of the U.S. Food and Drug Administration (MIC ≤2 mg/L denoting susceptibility and ≥8 mg/L denoting resistance). The presence of genes encoding β-lactamase, including CTX-M-type extended-spectrum β-lactamases (ESBLs), plasmid-borne AmpC β-lactamases, and carbapenemases, were investigated using primers previously described [10–14]. Polymerase chain reaction (PCR) amplicons were sequenced and the DNA sequences obtained were compared with those available in the NCBI GenBank database using BLAST searches.

Transfer of carbapenemase resistance, plasmid analysis, and bacterial genotyping

Conjugation experiment was carried out with *E. coli* J53 as the recipient to determine the transferability of the carbapenemase gene, as described previously [15]. Whole-cell DNA of clinical strains embedded in agarose gel plugs, digested with S1 nuclease, was separated by PFGE. Plasmids obtained by PFGE were transferred to nylon membranes and hybridized with digoxigenin-labeled $bla_{\text{NDM-1}}$-specific probes. Clonal relationships were analyzed using PFGE of *XbaI*-digested genomic DNA as previously described, and the results were analyzed according to the criteria proposed by Tenover et al. [16, 17]. Multilocus sequence typing (MLST) for these isolates was performed as described previously [18].

Clinical epidemiology

The clinical data were reviewed for each patient. Several parameters were assessed, including demographics, prior use of broad-spectrum antimicrobial agents, particularly carbapenems, and potential risk factors for infection or colonization with CRKP. Infection or colonization with CRKP was defined according to the definition of nosocomial infections from the Centers for Disease Control and Prevention (CDC) [19].

Results

Antimicrobial susceptibility testing and β-lactamase characterization

All 22 isolates were resistant to cephalosporin and β-lactam/β-lactamase inhibitor combinations, while none were resistant to tigecycline or colistin. Overall, 59.1, 77.3, and 100 % of these strains were resistant to imipenem, meropenem, and ertapenem, respectively. And 4.5 % (1/22), 4.5 % (1/22), and 90.9 % (20/22) were resistant to amikacin, ciprofloxacin, and aztreonam, respectively (Table 1). Seventeen isolates were $bla_{\text{NDM-1}}$-positive by PCR and DNA sequencing. Of the 17 $bla_{\text{NDM-1}}$-positive isolates, 47 % (8/17) co-harbored $bla_{\text{CTX-M-15}}$ and 5.9 % (1/17) co-harbored $bla_{\text{DHA-1}}$. No carbapenemase genes were detected among the remaining four isolates; however, 50 % (2/4) of isolates produced $bla_{\text{CTX-M-15}}$. Half (11/22) of the isolates carried class 1 integron, while sequence data showed that none has resistant gene cassettes. No other PCR products were obtained for any of the other genes investigated (Table 1).

Transfer of carbapenemase resistance, plasmid analysis, and bacterial genotyping

The 17 $bla_{\text{NDM-1}}$-positive *K. pneumoniae* isolates were selected for conjugation. The results of conjugation experiments indicated that the plasmids with $bla_{\text{NDM-1}}$ from 15 isolates were successfully transferred from donors to recipient *E. coli* J53, and the conjugants exhibited high resistance to carbapenems, consistent with the detection of $bla_{\text{NDM-1}}$. The MICs of imipenem, meropenem, and ertapenem for the conjugants ranged from 1 to 8 mg/L, and the antimicrobial susceptibility patterns of the conjugants were similar to their donors (Table 1). Hybridization analysis showed that the plasmids

Table 1 Antimicrobial susceptibilities and characteristics of the 22 carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains and their transconjugants (TC)

Isolate	Age/sex	Specimen	Ward	MIC (mg/L)					β-lactamase genes	MLST	Hospital stay (days)	Days to CRKP detection	Infection or colonization	Outcome
				IPM	MEM	ETP	COL	TGC						
KP1	17 days/F	Sputum	NICU	4	4	16	1	0.5	NDM-1	ST76	70	17	Colonization	Improvement
KP1-TC				2	2	4	0.25	0.25	NDM-1	/				
KP8	16 days/M	Sputum	NICU	1	2	16	0.5	0.5	NDM-1, CTX-M-15	ST76	55	16	Colonization	Improvement
KP8-TC				2	2	4	0.25	0.25	NDM-1	/				
KP29	30 day/F	Sputum	NICU	1	4	16	0.5	0.5	NDM-1, DHA	ST76	46	41	Infection	Improvement
KP29-TC				2	2	4	0.5	0.25	NDM-1	/				
KP36	12 years/M	Sputum	Specialty	16	16	32	0.5	0.5	NDM-1	ST76	4	1	Infection	Improvement
KP36-TC				2	2	4	0.5	0.5	NDM-1	/				
KP37	30 days/F	Sputum	Neonatal	16	16	32	0.5	0.5	NDM-1	ST76	19	1	Colonization	Improvement
KP37-TC				2	2	4	0.5	0.25	NDM-1	/				
KP42	21 days/M	Sputum	NICU	32	16	32	0.5	1	NDM-1, CTX-M-15	ST37	62	21	Infection	Improvement
KP42-TC				2	2	4	0.25	0.25	NDM-1	/				
KP45	2 months/M	Sputum	Abandoned	1	4	16	0.5	1	NDM-1, CTX-M-15	ST37	72	44	Infection	Death
KP45-TC				2	2	4	0.25	0.25	NDM-1	/				
KP51	30 days/F	Sputum	NICU	16	16	16	0.5	1	NDM-1, CTX-M-15	ST37	61	35	Infection	Death
KP51-TC				2	2	4	0.5	0.25	NDM-1	/				
KP58	12 days/M	Sputum	Neonatal	2	2	8	1	1	NDM-1, CTX-M-15	ST37	18	12	Infection	Improvement
KP58-TC				2	4	8	0.5	0.25	NDM-1	/				
KP65	10 years/M	sputum	Nephrology	8	16	32	1	1	NDM-1, CTX-M-15	ST37	51	28	Infection	Improvement
KP65-TC				2	2	4	0.5	0.25	NDM-1	/				
KP67	30 days/M	Sputum	NICU	1	2	8	0.5	0.5	NDM-1, CTX-M-15	ST37	43	36	Infection	Improvement
KP67-TC				1	2	4	0.5	0.25	NDM-1	/				
KP70	4 months/M	Urine	Outpatient	1	8	32	1	1	NDM-1-	ST846	/	/	/	Improvement
KP70-TC				2	2	8	0.25	0.25	NDM-1	/				
KP77	9 days/M	Endotracheal tube	NICU	2	2	8	0.5	1	NDM-1	ST37	19	10	Colonization	Improvement
KP77-TC				2	4	8	0.25	0.25	NDM-1	/				
KP83	17 days/M	Sputum	NICU	4	2	4	0.25	1	NDM-1, CTX-M-15	ST37	24	17	Colonization	Discharged
KP83-TC				2	4	8	0.5	0.25	NDM-1	/				

Table 1 (continued)

Isolate	Age/sex	Specimen	Ward	MIC (mg/L)					β-lactamase genes	MLST	Hospital stay (days)	Days to CRKP detection	Infection or colonization	Outcome
				IPM	MEM	ETP	COL	TGC						
KP94	24 days/F	Sputum	NICU	64	64	128	0.5	1	NDM-1, CTX-M-15	ST37	39	24	Colonization	Discharged
KP94-TC				2	4	4	0.5	0.25	NDM-1	/				
KP20	10 months/M	Sputum	CICU	2	4	16	0.5	1	–	ST571	42	27	Infection	Improvement
KP20-TC				2	2	8	0.5	0.25	–	/				
KP38	5 days/M	Sputum	Neonatal	8	16	32	0.5	1	–	ST76	6	1	Infection	Improvement
KP38-TC				4	4	8	0.5	0.25	–	/				
KP85	1 day/M	Sputum	NICU	16	16	32	0.5	1	CTX-M-15	ST37	31	27	Colonization	Discharged
KP85-TC				2	2	4	0.25	0.25	–	/				
KP97	5 days/M	Sputum	NICU	16	16	32	0.5	1	CTX-M-15	ST37	28	26	Colonization	Improvement
KP97-TC				2	2	2	0.5	0.25	–	/				
KP16	13 months/M	Pus	Surgery	4	128	128	0.5	1	KPC	ST11	14	1	Infection	Improvement
KP22	30 days/M	Sputum	Neonatal	16	16	32	0.5	0.5	NDM-1	ST76	6	1	Colonization	Improvement
KP53	6 months/M	Sputum	Cardiology	16	16	32	0.5	0.5	NDM-1	ST37	9	1	/	/
E. coli J53	/	/	/	0.25	≤0.06	≤0.06	0.5	0.25	–	/	/	/	/	

F female; *M* male; *MLST* multilocus sequence typing; *NICU* neonatal intensive care unit; *CICU* cardiac intensive care unit; *CRKP* carbapenem-resistant *Klebsiella pneumoniae*; *IPM* imipenem; *MEM* meropenem;, *ETP* ertapenem; *COL* colistin; *TGC* tigecycline

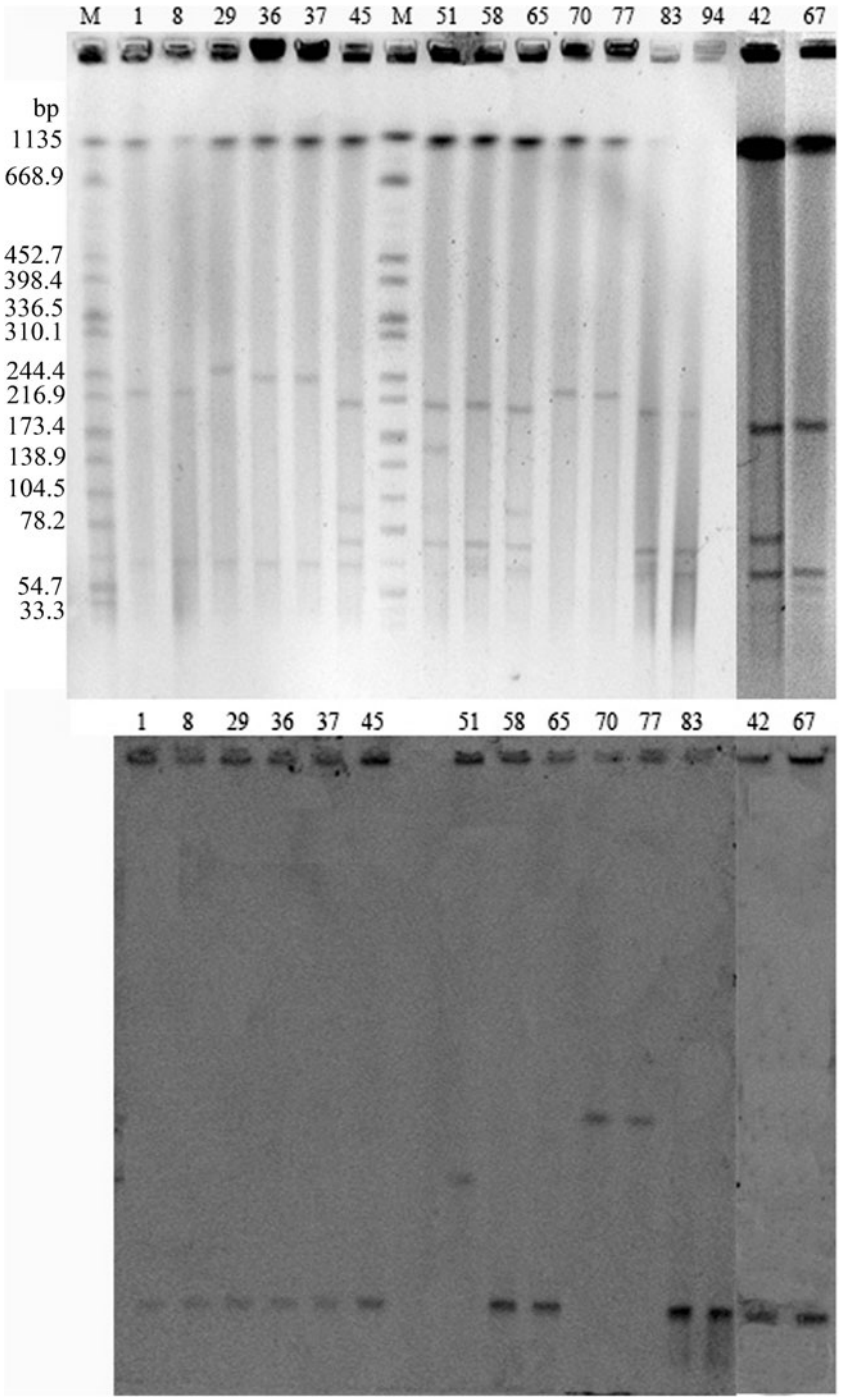

Fig. 1 S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) patterns (*top*) of 15 bla_{NDM-1}-positive *Klebsiella pneumoniae* and Southern hybridization (*bottom*). Lane M: marker (*Salmonella* H9812); lanes 1, 8, 29, 36, 37, 45, 51, 58, 65, 70, 77, 83, 94, 42, and 67: clinical strains

carrying the bla_{NDM-1} gene were approximately 50–240 kb in size (Fig. 1).

Five distinct PFGE patterns (PFGE types A–E) were observed among the 22 CRKP isolates: type A (11/22, 50 %), type C (8/22, 36.4 %), and one isolate each for type B, type D, and type E (Fig. 2). Five distinct MLST sequence types (STs) were observed among the 22 CRKP isolates, including ST76 ($n=8$), ST37 ($n=11$), ST846 ($n=1$), ST11 ($n=1$), and ST571 ($n=1$). Of the 17 bla_{NDM-1}-positive *K. pneumoniae* isolates, 16 from inpatients were identified as ST76 ($n=7$) and ST37 ($n=9$) and one isolate from an outpatient belonged to ST846. One bla_{KPC}-positive *K. pneumoniae* belonged to ST11 (Fig. 2).

Clinical epidemiology

The clinical details of the patients are shown in Table 1. The 22 isolates were identified in a diverse population of children.

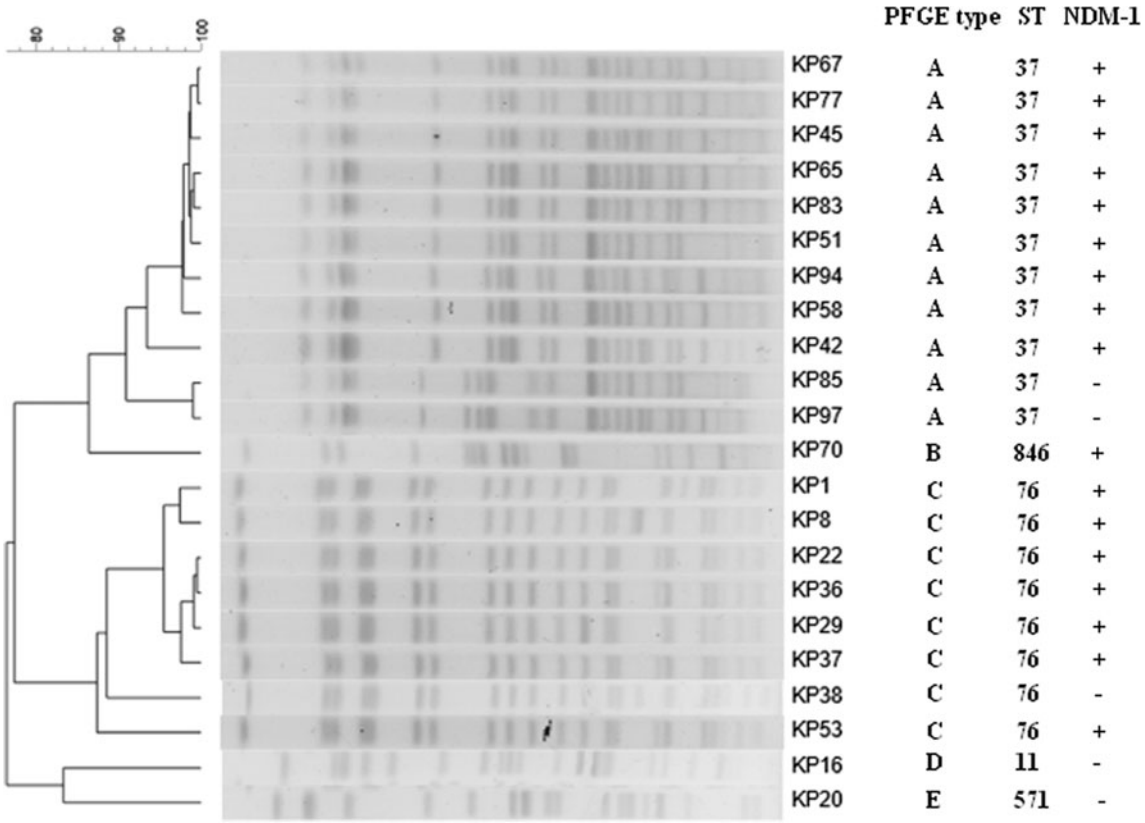

Fig. 2 DNA fingerprinting and multilocus sequence typing (MLST) of 22 carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates

The median age of the 22 children (17 males and 5 females) was 1 month (range, 1 day to 12 years). The most common underlying conditions were neonatal respiratory distress syndrome (27.3 %, 6/22), pneumonia and bronchopneumonia (31.8 %, 7/22), and neonatal asphyxia or perinatal asphyxia (13.6 %, 3/22). Indwelling devices were used in some patients, including endotracheal tube (45.5 %, 10/22), arteriovenous intubation (31.8 %, 7/22), mechanical ventilation (9.1 %, 2/22), urinary catheter (9.1 %, 2/22), and gastric tube (9.1 %, 2/22). Most patients (81.8 %, 18/22) were treated with antimicrobial agents, including ampicillin–sulbactam, cefotaxime, ceftriaxone, imipenem, and meropenem. Most patients were improved or discharged after treatment, except for two deaths (Table 1).

Discussion

Infectious diseases caused by NDM-1-producing isolates were known to be associated with significant morbidity and mortality, which was even worse among pediatric populations due to limited therapeutic options [20]. All the isolates were susceptible to tigecycline and colistin in our study; however, tigecycline is not recommended in children because of the risk of dental staining and, currently, colistin is not available for patients in China. Besides, aminoglycosides and fluoroquinolones are also restricted in children due to nephrotoxicity and ototoxicity. In the absence of effective antibiotic therapy, early monitoring of CRKP infection or colonization on admission may play a more important role for timely control of the spread of CRKP [21].

In our study, *K. pneumoniae* ST76 ($n=8$) and ST37 ($n=11$) were predominant epidemic clones. Although both STs did not belong to the most common NDM-1-positive clones (ST14 and ST11), *K. pneumoniae* ST37 has also been reported in India, the UK, and the USA, while *K. pneumoniae* ST76 bearing $bla_{\text{NDM-1}}$ was reported for the first time in this study [22]. The results of clinical epidemiology indicated that the risk factors for acquiring CRE isolates included invasive procedures (especially surgical operations), indwelling urinary catheters, change of sickbeds, and previous in-hospital cephalosporin use [23]. Our study indicated that immunodepression, invasive procedures, and prior use of broad-spectrum antibiotics might increase the chance of infection or colonization of CRE isolates. In our study, two pediatric patients died of *K. pneumoniae* ST37 infection, while no patients died of *K. pneumoniae* ST76 infection.

It is noteworthy that no carbapenemase resistance genes were detected in 18.2 % (4/22) of the CRKP strains. We suspect that a new mechanism, such as the presence of new metallo-β-lactamases or variants of certain carbapenemases,

might contribute to the resistance to carbapenems. Further studies are needed to confirm this point. In this study, we reported a nosocomial outbreak of bla_{NDM-1}-producing *K. pneumoniae* ST37 and ST76 in neonates. Although two outbreaks involving *K. pneumoniae* ST17 and ST20 were reported recently, to our knowledge, this is the first report of an NDM-1-producing *K. pneumoniae* ST37 and ST76 outbreak [24, 25]. The vulnerability to colonization or infection with CRE isolates among pediatric patients highlights the necessity of intervention with strict infection-control measures, including proper hand hygiene, contact precautions, and cohort nursing care, to reduce the cross-infection and avoid the rapid spread or clonal dissemination of carbapenemase-producing Enterobacteriaceae strains in healthcare facilities.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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