Dissemination of IMP-4-encoding pIMP-HZ1-related plasmids among Klebsiella pneumoniae and Pseudomonas aeruginosa in a Chinese teaching hospital

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A total of 26 blaIMP-4-carrying strains of Pseudomonas aeruginosa and Klebsiella pneumoniae were isolated from 2009 to 2013 in a Chinese teaching hospital, and these strains can be assigned into multiple sequence types or allelic profiles as determined by multilocus sequence typing. Of these strains, P. aeruginosa P378 and K. pneumoniae 1220 harbor the IMP-4-encoding plasmids pP378-IMP and p1220-IMP, respectively, whose complete nucleotide sequences are determined to be genetically closely related to the IncN1-type plasmid pIMP-HZ1. pP378-IMP/p1220-IMP-like plasmids are hinted to be present in all the other blaIMP-4-carrying strains, indicating the dissemination of pIMP-HZ1-related plasmids among K. pneumoniae or P. aeruginosa of different genotypes in this hospital. pP378-IMP carries two distinct accessory resistance regions, a blaIMP-4-carrying class 1 integron In823b, and a truncated Tn3-family unit transposon ΔTn6292-3′ harboring the quinolone resistance gene qnrS1.

Massive fragmentation and rearrangement of these accessory genetic contents occur among p1220-IMP and IMP-HZ1 relative to pP378-IMP. blaIMP-4 is also present in the In823b remnants from p1220-IMP and IMP-HZ1, while qnrS1 is located in a Tn6292-derive fragment from pIMP-HZ1 but not found in p1220-IMP. pP378-IMP represents the first fully sequenced IncN-type plasmid from P. aeruginosa.

Plasmids belonging to the IncN incompatibility group commonly have broad host range and high transmission efficiency, and they are important to the dissemination of clinically important resistance determinants among enterobacterial species. Location of the major carbapenem resistance genes such as blaIMP-1, blaKPC-2, blaNDM-1 and blaVIM-1 have been found on different IncN-type plasmids. The IncN plasmids can be further divided into three subgroups, namely IncN1to IncN3, with their reference plasmids R46 (accession number AY046276), p271A5 and pN-Cit6, respectively. These three different plasmid subgroups have similar plasmid scaffolds but limited nucleotide sequence similarity over their backbones5,6.

The IMP-type enzymes are among the clinically most important metallo-β-lactamase and can hydrolyze almost all β-lactams including carbapenems. The first IMP-type enzyme IMP-1 was described in 1991 in Japan from Serratia marcescens7 and, since then, at least 52 IMP-variant enzymes (http://www.ncbi.nlm.nih.gov/projects/pathogens/beta-lactamase-data-resources/) have been reported worldwide among Enterobacteriaceae, Acinetobacter, and Pseudomonas species. The blaIMP genes are commonly located on a plasmid-borne class 1 integrons, which are critical for the acquisition, maintenance, and dissemination of resistance in gram-negative organisms.

Up to now, a total of three blaIMP-carrying IncN1 plasmids, namely pKPI-61, and pIMP-HZ19 and its isoform pIMP-1495 (GenBank accession number KM977631), all of which are recovered from Klebsiella pneumoniae,
Figure 1. Prevalence of blaIMP among imipenem-nonsusceptible bacterial isolates. The blaIMP genes are screened by PCR\(^2\), followed by amplicon sequencing. All the detected blaIMP markers are the blaIMP\(_4\) gene.

have been fully sequenced. pIMP-HZ1/pIMP-1495 and pKPI-6 harbor the blaIMP\(_4\) and blaIMP\(_6\) genes captured by two distinct class 1 integrons In823 and In722, respectively.

This study provides the evidence for dissemination of blaIMP\(_4\)-carrying pIMP-HZ1-related plasmids among K. pneumoniae or P. aeruginosa strains of different genotypes from 2009 to 2013 in a Chinese public hospital. The whole genome sequences of pP378-IMP and p1220-IMP from two of these strains are determined to be genetically closely related to the IncN1-type plasmid pIMP-HZ1. pP378-IMP contains a blaIMP\(_4\)-carrying integron In823b and a truncated Tn3-family unit transposon \(\Delta \text{Tn6292-3'}\) harboring qnrS1; by contrast, massive fragmentation of In823b and Tn6292 and further complex rearrangement of the relevant fragments occur in p1220-IMP (containing blaIMP\(_{37}\) and qnrS1) and pIMP-HZ1 (containing only blaIMP\(_4\)). Denoting dramatic genetic variations in the accessory resistance regions among these three plasmids.

Results and Discussion

blaIMP\(_4\)-carrying K. pneumoniae and P. aeruginosa isolates. From 2009 to 2013, a total of 403 imipenem-nonsusceptible strains of K. pneumoniae (53 strains), P. aeruginosa (164 strains) and A. baumannii (186 strains) were isolated from the patients (with infections at various sites of their bodies) from our hospital (Fig. 1). Presence of blaIMP was detected by PCR in 19 (35.85\%) strains of K. pneumoniae and in 7 (4.27\%) strains of P. aeruginosa (Fig. 1), and all these detected blaIMP genes were blaIMP\(_4\) as further determined by sequencing. None of the A. baumannii strains tested by PCR harbored the blaIMP marker (Fig. 1).

These blaIMP\(_4\)-carrying K. pneumoniae and P. aeruginosa isolates (Table S1), scattered from 2009 to 2013, came from five distinct specimens (sputum, lung lavage fluid, wound secretion, urine, and blood) from eight different departments (Department of Pediatrics, Department of Pediatric ICU, Department of Respiratory Medicine, Department of Neurology, Department of Neurosurgery, Department of Cerebral Surgery, Department of Pediatrics, and Department of emergency).

As determined by multilocus sequence typing (MSLT), the blaIMP\(_4\)-positive K. pneumoniae strains could be assigned into six different sequence types (STs), namely ST37 (allelic profile: 2-9-2-1-13-16-1), ST107 (2-1-2-17-27-39-1), ST133 (12-1-1-2-5-36), ST323 (2-1-1-9-93), ST686 (4-1-1-3-3-54), and ST1114 (4-3-2-1-10-17) (Table S1). The blaIMP\(_4\)-positive P. aeruginosa strains could be assigned into at least four allelic profiles, namely 15-?-1-4-11-4-10, 6-?-4-3-11-4-7, 2-?-5-1-3-6-11, 111-?-64-30-26-59-7, but unfortunately they could not be assigned into any of known or novel STs because the \(\text{aroE}\) sequences (corresponding to '?' in the allelic profiles) for all the strains tested could not be obtained with repeated attempts (Table S1). The above results indicated the non-clonal dissemination of blaIMP\(_4\)-carrying K. pneumoniae and P. aeruginosa in the hospital.

pP378-IMP and p1220-IMP from P. aeruginosa and K. pneumoniae. Two blaIMP\(_4\)-positive strains, P. aeruginosa P378 isolated from the urine specimen of a 36-year-old male with urinary tract infection and conscious disturbance, and K. pneumoniae 1220 from the blood specimen of a three-month-old baby boy with neonatal septicemia and hyperbilirubinemia, were arbitrarily selected for transferring the blaIMP\(_4\) marker into E. coli EC600 through conjugation, generating the blaIMP\(_4\)-positive E. coli transconjugants P378-IMP-EC600 and 1220-IMP-EC600, respectively. All these four strains had the class B carbapenemase activity and were resistant to piperacillin, piperacillin/tazobactam, cefazolin, cefuroxime, ceftazidime, cefepime, imipenem, and meropenem; moreover, P378 and P378-IMP-EC600, but not 1220 and 1220-IMP-EC600, were resistant to ciprofloxacin and levofloxacin (Table 1). Taken together, either P. aeruginosa P378 or K. pneumoniae 1220 harbors a conjugal blaIMP\(_4\)-carrying plasmid, designated pP378-IMP and p1220-IMP, respectively, which account for the carbapenem resistance phenotype.

Whole-genome sequencing of pP378-IMP and p1220-IMP (with mean coverage >80), recovered from the P378-IMP-EC600 and 1220-IMP-EC600 strains, respectively, showed that these two plasmids have circularly closed DNA sequences, 51,207 bp and 46,629 bp in length, respectively (Fig. 2). pP378-IMP and p1220-IMP have mean GC contents of 50.5\% and 50.7\% and contain 64 and 60 predicted open reading frames in total, respectively (Fig. 2).
The entire sequences of pP378-IMP and p1220-IMP are mostly similar to that of pIMP-HZ1 (>99% query coverage and maximum >99% nucleotide identity). pP378-IMP, p1220-IMP and pIMP-HZ1 possess the conserved IncN1-type backbone regions, which contain a repA gene and its iterons (RepA-binding sites; regulation of replication) for plasmid replication, the tra genes and kikA-korB for conjugal transfer, the CUP (conserved upstream repeat) -controlled regulon, the stbABC-orfD operon, and resP) for plasmid maintenance (Fig. 2). These backbone regions are highly similar to the IncN1 prototype plasmid R46 from Salmonella enterica serovar Typhimurium.

There are four major genetic differences among the backbones of pP378-IMP, p1220-IMP and IMP-HZ1. First, a total of 7 copies of 37 to 40 bp tandem repeats are observed within the repA iterons of pIMP-HZ1, while only 3 copies are found in pP378-IMP and p1220-IMP (Fig. 3). Second, pP378-IMP and IMP-HZ1 contains an intact antirestriction system ecoRII-ecoRIImet (located around 8.5 kb to 11.5 kb nucleotide position of pP378-IMP), while only a truncated ecoRIImet gene is found in p1220-IMP and this truncation likely results from the insertion of ISKpn19 upstream (Fig. 3). Third, the inversion of the conjugal transfer region from orf207 to the 3'-end remnant of fipA undergone occurs within pP378-IMP and p1220-IMP related to IMP-HZ1 (Fig. 3).

### Table 1. Antimicrobial drug susceptibility profiles.

| Category | Antibiotics | MIC (mg/L)/antimicrobial susceptibility |
|----------|-------------|-----------------------------------------|
| Penicillins | Ampicillin | ≥32R | ≥32R | ≥32R | ≥32R | 85 |
| | Ampicillin/sulbactam | ≥32R | ≥32R | ≥32R | ≥32R | 45 |
| Cephalosporins | Cefazolin | ≥64R | ≥64R | ≥64R | ≥64R | ≤45 |
| | Cefuroxime | ≥64R | ≥64R | ≥64R | ≥64R | 161 |
| | Cefotetan | ≥64R | ≥64R | ≥64R | ≥64R | ≤45 |
| | Ceftriaxone | ≥64R | ≥64R | ≥64R | ≥64R | ≤15 |
| | Cefazidime | ≥64R | ≥64R | ≥64R | ≥64R | ≤15 |
| | Cefepime | 32R | 32R | ≥64R | 32R | ≤15 |
| Carbapenems | Imipenem | 8R | 8R | 8R | 8R | ≤1S |
| | Meropenem | 8R | 8R | 8R | 8R | ≤0.25S |
| Fluoroquinolones | Ciprofloxacin | ≤0.25S | ≤0.25S | ≥4R | ≥4R | ≤0.25S |
| | Levofloxacin | 0.5S | 0.5S | ≥8R | ≥8R | 0.5S |
| | Gentamicin | ≤1S | ≤1S | ≤1S | ≤1S | ≤1S |
| | Tobramycin | ≤1S | ≤1S | ≥16R | ≥16R | ≤1S |
| Aminoglycosides | Amikacin | ≤2S | ≤2S | 16S | ≤2S | ≤2S |
| | Gentamicin | ≤1S | ≤1S | ≤1S | ≤1S | ≤1S |
| | Tobramycin | ≤1S | ≤1S | ≥16S | ≥16R | ≤1S |
| Sulfanilamides | Trimethoprim/sulfamethoxazole | ≤20S | ≤20S | ≥320R | ≤20S | ≤20S |

S = sensitive; R = resistant; I = intermediate.

Figure 2. Schematic maps of sequenced plasmids. Genes are denoted by arrows and colored based on gene function classification. The innermost circle presents GC-Skew [(G−C)/(G + C)] with a window size of 500 bp and a step size of 20 bp. The blue circle presents GC content. Shown also are backbone and accessory module regions.

Backbones of pP378-IMP and p1220-IMP. The entire sequences of pP378-IMP and p1220-IMP are mostly similar to that of pIMP-HZ1 (>99% query coverage and maximum >99% nucleotide identity). pP378-IMP, p1220-IMP and pIMP-HZ1 possess the conserved IncN1-type backbone regions, which contain a repA gene and its iterons (RepA-binding sites; regulation of replication) for plasmid replication, the tra genes and kikA-korB for conjugal transfer, the CUP (conserved upstream repeat) -controlled regulon, the stbABC-orfD operon, and resP) for plasmid maintenance (Fig. 2). These backbone regions are highly similar to the IncN1 prototype plasmid R46 from Salmonella enterica serovar Typhimurium.

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The fourth major genetic difference (Fig. 4) is found within the CUP-controlled regulon 10. A total of four putative operons, namely the CUPA operon, the CUPB operon, the CUP5 operon, the CUP4 operon, the CUP3 operon, the CUP2 operon and the CUP1 operon, are arranged within this regulon; each of these operons contains a putative ArdK-binding site and a promoter, which are responsible for ArdK-dependent expression of corresponding genes 10. Compared with pIMP-HZ1, the translocation of the CUP2 operon occurs within pP378-IMP and p1220-IMP, which most likely results from the homologous recombination mediated by CUP1, CUP2 and CUP4 (Fig. 4).

Accessory modules of pP378-IMP and p1220-IMP. pP378-IMP carries three separate accessory modules, a 6492 bp class 1 integron designated In823b, a 290 bp IS1 remnant, and a 7075 bp truncated version (designated ΔTn6292-3′) of a presumed Tn3-family unit transposon Tn6292 (Fig. 5). The 290 bp IS1 remnant, which contains only ΔinsB (transposase) and inverted repeat right (IRR) and is inserted between ecoRIImet and orf207, is shared by pP378-IMP, p1220-IMP and IMP-HZ1 (Fig. 1). Multiple copies of IS26 are present in the In823b- and Tn6292-related regions of pP378-IMP, p1220-IMP and IMP-HZ1, and the common component IS26 would act as an adaptor 11,12 to mediate massive fragmentation and rearrangements of In823b- and Tn6292-related regions in p1220-IMP and IMP-HZ1 relative to pP378-IMP (Fig. 5), leaving different mosaic assemblies from the remnants of In823b and Tn6292 in p1220-IMP and IMP-HZ1. Nevertheless, all these accessory genetic contents are integrated at two “hotspots” (Fig. 1), namely a region downstream of resP (resolvase) and a region within fipA (fertility inhibition protein), which has been previously described in IncN1 plasmids 2,4.

Compare with the fragmentary In823b-related regions in pIMP-HZ1 and p1220-IMP, the In823b integron from pP378-IMP looks like a primitive form flanked by a complete set of inverted repeats (IRs, 25 bp in length) and direct repeats (DRs, 5 bp in length: target site duplication signals of transposition) (Fig. 5)13. The 5′-conserved segment [5′-CS: IRi (inverted repeat initial)-intl1 (integrase)-attl] of In823b is disrupted by the insertion of IS26 into intl1. In823b contains a single resistance gene cassette blaIMP-4-attCblaIMP-4, and a group IIc intron Kl.pn.I3 disrupts an unusual attC site that appears to be a chimera between attCblaIMP-4 and attCdfrA14GC. Downstream of attCdfrA14GC is a structure mobC (Mobilization protein)-IRi-IS6100-IRt (inverted repeat terminal), but the typical 3′-conserved segment [3′-CS: qacED1 (quaternary ammonium compound resistance)-sulI (sulfonamide resistance)-IRt] is not found. The expression of blaIMP-4 is driven by a single promoter PcWTGN-10 which is a derivative of the weak promoter PcW and much stronger than PcW due to the C to G mutation 2 bp upstream of the −10 element 14.

Two Tn6292-related fragments (namely ΔTn6292-3′ and ΔTn6292-5′) are present in pP378-IMP and pIMP-HZ1, respectively, with a large overlapping region between these two plasmids, which promotes us to propose a prototype Tn3-family unit transposon Tn6292, 7314 bp in length, with typical 38 bp IRs (IRL: inverted repeat left; IRR: inverted repeat right) at both ends (Fig. 5). The Tn6292 core transposition module tnpA (transposase)-res (resolution site)-tnpR (resolvase) is disrupted by the insertion of ISKpm19 into tnpA, leaving it truncated

**Figure 3.** Linear comparison of sequenced plasmids. Genes are denoted by arrows and colored based on gene function classification. Shading regions denote regions of homology (>95% nucleotide identity).

**Figure 4.** CUP-control regulons Genes are denoted by arrows and colored based on gene function classification. The broken lines with terminal arrows indicate the core promoter regions of indicated operons. Shading regions denote shared DNA regions of homology (>95% nucleotide identity).
and broken into two separate parts ΔtnpA-3′ and ΔtnpA-5′; downstream of tnpR is a quinolone resistance region qnrS1-ΔISEcl2-ΔIS26-orf198. The qnrS1 gene and its upstream insertion sequence ΔISEcl2 constitute a core qnrS1 genetic platform that is widely found in resistance plasmids from Enterobacteriaceae species, and it is thought that ΔISEcl2 could have played a role in the original acquisition of qnrS1. The ΔTn6292-3′ element of pP378-IMP is a 7075 bp 3′-region of Tn6292 lacking IRL-ΔtnpA-3′, while ΔTn6292-5′ from pIMP-HZ1 is a 6048 bp 5′-region of Tn6292 in the absence of ΔIS26-orf198-IRR (Fig. 5).

Compared to In823b and Tn6292-3′ from pP378-IMP, massive fragmentation of these two accessory regions, followed by further inversion and translocation of the resulting In823b- and Tn6292-derived fragments, occurs in p1220-IMP and IMP-HZ1, leaving the assembly of different combinations of accessory regions with a very complex mosaic nature in these two plasmids (Fig. 5). pP378-IMP contains a total of two resistance genes blaIMP-4 and qnrS1, which are captured by In823b and ΔTn6292-3′, respectively. blaIMP-4 is also present in the In823b-derived elements In823b-1 and In823b-2 (which can be discriminated as the partial regions of In823b) from p1220-IMP and IMP-HZ1, respectively. qnrS1 is also present in ΔTn6292-5′ from pIMP-HZ1, but it not found in p1220-IMP.

**Prevalence of pP378-IMP/p1220-IMP-related plasmids.** A total of 12 backbone genes repA, mrr, kikA, traL, traB, traF, trAI, stdB, ccgAII, ardA, mucB, and ardK as well as the accessory quinolone-resistance gene qnrS1 were arbitrarily selected for PCR detection, followed by amplicon sequencing (data not shown). It was found that all these 12 backbone genes were present in all the blaIMP-4-carrying 19 K. pneumoniae strains and 7 P. aeruginosa strains (Table S1). The above results indicated that pP378-IMP/p1220-IMP-like plasmids were harbored in all these blaIMP-4-carrying K. pneumoniae strains and P. aeruginosa strains (Table S1), denoting the probable coexistence of the In823-derived blaIMP-4 regions and the Tn6296-derived qnrS1 regions in these strains.

**Methods**

**Bacterial strains and identification.** Bacterial species was identified by 16S rRNA gene sequencing and by PCR detection of K. pneumoniae-specific gene kih16, P. aeruginosa-specific aafA19 and A. baumannii-specific blaOXA-5120. The major plasmid-borne carbapenemase and extended-spectrum β-lactamase genes were screened for by PCR, followed by amplicon sequencing on ABI 3730 Sequencer (LifeTechnologies, CA, USA). The MLST schemes for K. pneumoniae and P. aeruginosa were derived from the PubMLST database (http://pubmlst.org/).

**Plasmid conjugal transfer.** Plasmid conjugal transfer experiments were carried out with the rifampin-resistant Escherichia coli EC600 (LacZ-, NalR, RifR) being used as recipient and strain P378 or 1220 as donor. 3 ml of overnight culture of each of donor and recipient bacteria were mixed together, harvested
and resuspended in 80 μl of Brain Heart Infusion (BHI) broth (BD Biosciences). The mixture was spotted on a 1 cm² filter membrane that was placed on BHI agar (BD Biosciences) plate, and then incubated for maturing at 37 °C for 12 to 18 h. Bacteria were washed from filter membrane and spotted on Muller-Hinton (MH) agar (BD Biosciences) plate containing 1000 μg/ml rifampin and 2 μg/ml imipenem for selection of blaIMP-positive E. coli transconjugants.

Detection of carbapenemase activity. Activity of class A/B/D carbapenemases in bacterial cell extracts was determined via a modified CarbaNP test. Overnight bacterial cell culture in MH broth was diluted 1:100 into 3 ml of fresh MH broth, and bacteria were allowed to grow at 37 °C with shaking at 200 rpm to reach an OD₆₀₀ of 1.0 to 1.4. If required, ampicillin was used at 200 μg/ml. Bacterial cells were harvested from 2 ml of the above culture, and washed twice with 20 mM Tris-HCl (pH 7.8). Cell pellets were resuspended in 500 μl of 20 mM Tris-HCl (pH 7.8), and lysed by sonication, followed by centrifugation at 10000 × g for 5 min. 50 μl of the supernatant (the enzymatic bacterial suspension) were mixed with 50 μl of substrate I to V, respectively, followed by incubation at 37 °C for a maximum of 2 h. Substrate I: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8). Substrate II: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), and 0.6 mg/ml imipenem. Substrate III: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), 0.6 mg/ml imipenem, and 0.8 mg/ml tazobactam. Substrate IV: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), 0.6 mg/ml imipenem, and 3 mM EDTA (pH 7.8). Substrate V: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), 0.6 mg/ml imipenem, 0.8 mg/ml tazobactam, and 3 mM EDTA (pH 7.8).

Bacterial antimicrobial susceptibility test. Bacterial antimicrobial susceptibility was tested by VITEK 2 (BioMérieux Vitek, Hazelwood, MO, USA) and interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines.

Plasmid sequencing and sequence assembly. Plasmid DNA was isolated from E. coli transconjugant using Qiagen large construct kit (Qiagen, Hilden, Germany), and sequenced by whole-genome shotgun strategy using Qiagen large construct kit (Qiagen, Hilden, Germany), and sequenced by whole-genome shotgun strategy (http://arpcard.mcmaster.ca), BacMet (http://bacmet.biomedicine.gu.se/), and further annotated by BLASTP and BLASTN against UniProtKB/Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html) and NCBI NR databases. Annotation of resistance genes, mobile elements and other gene futures was based on the relevant databases including CARD (http://arpcard.mcmaster.ca), BacMet (http://bacmet.biomedicine.gu.se/), β-lactamases Database (http://www.ncbi.nlm.nih.gov/pathogens/submit_β_lactamase), ISfinder (https://www.is.biotoul.fr/), ISCR Elements Databases (http://medicine.cf.ac.uk/infect-immun/research/infection/antibacterial-agents/iscr-elements), INTEGRALL (http://integ rall.bio.uab.cat/), Tn Number Registry (http://www.ucl.ac.uk/eastman/research/departments/microbial-diseases/tn), and Group II Introns Databases (http://webapp2.scripps.edu/~groupii/bl ast.html). Sequence comparison was performed with BLASTN and CLUSTALW, and gene organization diagrams were drawn with Inkscape (https://inkscape.org). The complete sequence of pP378-IMP and p1220-IMP were submitted to GenBank under accession numbers KX711879 and KX711880, respectively.

Sequence annotation and genome comparison. The open reading frames and pseudogenes were predicted with GeneMarkS™ (http://topaz.gatech.edu/GeneMark), RAST (http://rast.nmpdr.org/), and Prodigal (http://compbio.ornl.gov/prodigal), and further annotated by BLASTP and BLASTN against UniProtKB/Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html). The gaps were filled through combinatorial PCR and Sanger sequencing on ABI 3730 Sequencer.

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Author Contributions
D.Z., F.S. and P.X. designed experiments. W.F., F.S., W.L., Q.S., Q.W. and D.Z. performed experiments. D.Z., W.F., F.S., Q.S., Y.T. and W.C. analyzed data. W.F., F.S., Q.S., Y.T., W.C. and D.Z. contributed reagents, materials and analysis tools. D.Z., W.F., F.S. and P.X. wrote this manuscript.

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