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Characterization of multidrug-resistant *Klebsiella pneumoniae* isolated from the Chinese cobra *Naja atra* in a Beijing suburb

Haifeng WANG[1, 2, 3]; Hongxuan HE[2]

[1] School of Environmental Engineering, Central Plains Specialty Food Engineering &Technology Research Center, Yellow River Conservancy Technical Institute, Dong Jing Avenue, Kaifeng, China

[2] National Research Center for Wildlife-Borne Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, China

[3] Kaifeng Key Laboratory of Food Quality Assessment , Kaifeng, China

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Abstract: The emergence and spread of antibiotic resistance genes among Bacteria are a serious threat to global health. Their occurrence in animals which are in contact with humans is also important. The Chinese cobra (*Naja atra*, Elapidae), though a highly venomous species, is appreciated as food and as a source of materials used in traditional Chinese medicine. We are here reporting the isolation of multidrug-resistant *Klebsiella pneumoniae* (Enterobacteriaceae) from the lung of *Naja atra,* obtained from a snake farm in a Beijing suburb. Our study analyzed, using gene sequencing, the occurrence of antibiotic resistance genes (ARGs) in three *K. pneumoniae* isolates from two snakes. In addition, bacterial clones were identified by biochemical tests and phylogenetic analysis. Tests of antimicrobial susceptibility showed that all *K. pneumoniae* isolates were resistant to a host of antibiotics (piperacillin, cefazolin, gentamicin, tetracycline, doxycyclin, ciprofloxacin, levofloxacin, lomefloxacin, ofloxacin, norfloxacin, nalidixic acid, chloramphenicol, nitrofurantoin, sulfamethoxazole, and sulfamethoxazole/trimethoprim) but were susceptible to cefotaxime, cefixime, aztreonam, bramycin, amikacin, kanamycin, netilmicin, and streptomycin. Eighteen ARGs were detected in total DNA extracted from the isolates. Results showed three quinolone resistance genes (*oqxA, oqxB, qnrB*), the gyrA gene that confers resistance to beta-lactam antibiotics, and the emerging aac(3)-II gene that confers resistance to aminoglycosides. *K. pneumoniae* is an important opportunistic human pathogen and the emergence of multidrug-resistant *K. pneumoniae* in *N. atra* suggests the increasing risk of pathogen transmission between humans, livestock, and wildlife. Given the close association between foodborne pathogenic microorganisms and humans, it is key factor to identify these antibiotic resistance genes profile thereby minimize the risk of *K. pneumoniae* transmission.

Introduction

Klebsiella pneumoniae (Enterobacteriaceae) is an important opportunistic pathogen. It causes infection in the respiratory tract, bloodstream, septicemia, meningitis, soft tissue infections, abdominal cavity, and urinary tract (Du *et al.*, 2014; Jin *et al.*, 2015). The occurrence of *K. pneumoniae* in animals which are in contact with humans is important. The Chinese cobra (*Naja atra*, Elapidae) is a highly venomous species, which is appreciated as food and as a source of materials used in traditional Chinese medicine and it is farm-raised in China. There is a paucity of data on the bacterial associations of reptiles in general, and of the Chinese cobra in particular (e.g. Shek *et al.*, 2009) and there are no available reports on the antibiotics' susceptibility and resistance genes of *K. pneumoniae* associated to the Chinese cobra, *N. atra*.

In humans, the emergence of antimicrobial-resistant *K. pneumoniae* strains has been described in several countries (Doud *et al.*, 2009; Struelens *et al.*, 2010; Taitt *et al.*, 2017; Yamane *et al.*, 2004; Yong *et al.*, 2009). A recent study in China has shown the association of aminoglycoside resistance to the emergence of *armA*, *rmtB*, *aac (3)-II*, and *aac (6')-Ib* genes in *K. pneumoniae* isolates (Liang *et al.*, 2015). In addition, *cmlA*, *OXA-10*, *sul2*, and *qnrB* were reported from isolates of *K. pneumoniae* in Japan (Okade *et al.*, 2014).

The widespread expression of multiple resistance genes such as *qnrB1* and *qnrB12* genes, mutations in *gyrA* and *ramR*, as well as the expression of efflux pumps in isolates of *K. pneumoniae* carrying *blaKPC-2* has been reported (Aml *et al.*, 2017).

* Address correspondence to: Haifeng Wang, wanghaifeng@yrcti.edu.cn; Hongxuan He, hehx@ioz.ac.cn

The aim of this study was to investigate the pathogenicity, antimicrobial susceptibility, and resistance genes of *K. pneumoniae* isolates obtained from the Chinese cobra.

Material and Methods

Sample collection and identification

The samples were obtained from the lung of two adult individuals of the Chinese cobra, *N. atra,* obtained in a snake farm from Beijing suburbs. Inoculation loop samples were cultured on Mueller-Hinton agar medium at 37°C for 18 h. *K. pneumoniae* clones were biochemically identified and expanded in Mueller-Hinton liquid medium, using BD Phoenix™ 100 (Maryland, USA). DNA was extracted from the clones using genomic and plasmid extraction kits (Tiangen, China), and 16S rDNA gene PCR amplification was performed with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R (5'-GGTTACCTTGTTACGACTT-3') (Macrae *et al.*, 2000). PCR conditions were as follows: 95°C for 5 min, then 95°C for 30 s, 55°C for 2 min, 32 cycles, followed by 72°C for 7 min. The expected sizes of PCR products were confirmed on 1% agarose gels with ethidium bromide.

Infectivity of filtrates of lung homogenates on cell cultures

BHK21 (BabyHamster Syrian Kidney), MDCK (Madin-Daby canine kidney cells), Vero (Verda Reno) cell lines were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 10% fetal bovine serum (FCS, Gibco), in a humidified 5% CO_2 and 95% air at 37°C. Supernatant of lung homogenates from *N. atra,* passed through a 0.22 μm filter, were inoculated on BHK-21, MDCK, and VERO cells for 5 days, respectively.

Phylogenetic analyses

Sequencing was performed as a service by the Beijing Genomics Institute (BGI) and were compared to sequences deposited in the GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/). Alignments of bacterial clones and reference sequences were created using Clustal X. Phylogenetic analyses were performed by the neighbor-joining method, with 1,000 bootstraps using MEGA 6 following the Kimura 2-parameter model (Tamura *et al.*, 2007).

Mice inoculations

Five BALB/C mice per group were inoculated intraperitoneally with 0.2 mL (3×10^8 colony-forming units [CFU]/mL) of pure bacterial culture per 10g body weight, or with 0.2 mL of phosphate buffer, as control. Generally accepted ethical guidelines for the care and use of experimental animals were followed, and the experimental procedures were approved by the Ethics Committee on Animal Experimentation of the Institute of Zoology, Chinese Academy of Sciences. BD Phoenix™ 100 and 16SrDNA sequences were used for bacterial identication. Mice were observed during 14 days.

Antibiotics susceptibility testing

The antibiotics susceptibility analysis to different antimicrobial drug were determined using disk diffusion test according to CLSI (Clinical and Laboratory Standards Institute) recommendations and instructions.

Detection of resistance genes

PCR detection and gene identification were carried out for *AAC(3)-II*, *cmlA*, *CTX-M-l*, *gyrA*, *gyrB*, *blaKPC*, and *NDM-1*, (Kim *et al.*, 2009; Liu *et al.*, 2008; Yigit *et al.*, 2001; Yu *et al.*, 2007) in template DNA extracted from pure bacterial cultures. The primers used are shown in Tab. 1. The obtained sequences were compared to NCBI database. PCR was performed in a ABI Veriti96 (Life Technology) with PCR master mix (CWBIO). PCR conditions were as follows: 95°C for 5 min, then 95°C for 30 s, 60°C for 1 min, 40 cycles, followed by 72°C for 7 min. Reactions were performed in a 20 μL volume containing 10 μL PCR master mix, 8.5 μL double-distilled water, 1 μL forward and reverse primers, and 0.5 μL DNA template. The expected sizes of PCR products were confirmed on 1.5% agarose gels with ethidium bromide.

Results

Identification of K. pneumoniae

Purified amplification products of 16S rDNA gene were of the expected size (~1500 bp) and showed good specificity that only one specific DNA fragment appears. Isolates were confirmed to be *K. pneumoniae*, according to 16S rDNA sequences and BD Phoenix™ 100 testing.

Lack of cytopathogenic effects of filtered lung homogenates

Filtered supernatants of lung homogenate from *N. atra* did not cause any discernible alteration of 5 days cultures of BHK-21, MDCK, and VERO cells (Fig. 1).

Phylogenetic analysis

Three *K. pneumoniae* isolates (K1, K2, K3) were obtained in the current study, and they showed the same DNA sequence, which was similar to GenBank entries KJ845719 and KM035400. KJ845719 came from Yunnan Animal Science and Veterinary Institute of China through direct submission to NCBI (National Center of Biology and Information) and corresponded to a highly virulent *K. pneumoniae*. KM035400 was isolated from human fecal matter in India (Fig. 2).

Effects of K. pneumoniae *inoculation in mice*

Twenty four hours after inoculation, the mice became non reactive, with appetite loss, and they developed quivering, weight loss and expiratory dyspnea. The three experimental mice died within 72 h after inoculation, while the control mice survived normally. On necropsy, an enlarged spleen with bleeding as well as severe intestinal edema were found (Fig. 3). *K. pneumoniae* was isolated from spleens of dead mice, as identified by BD Phoenix™ 100 and 16SrDNA sequences.

TABLE 1
Resistance genes: Primer sequences, amplicon sizes and annealing temperatures used in PCR assays

Gene Symbol	Sequence (5'-3')	Amplicon (bp)	Annealing (°C)
AAC(3)-II	GGCGACTTCACCGTTTCT	412	52
	GGACCGATCACCCTACGAG		
cmlA	GGGTGGCGGGCTATCTTT	467	52
	GCGACACCAATACCCACTAG		
CTX-M-l	CAGCGCTTTTGCCGTCTAAG	94	52
	GGCCCATGGTTAAAAAATCACTC		
gyrA	CGATGTCGGTCATTGTTG	496	52
	ACTTCCGTCAGGTTGTGC		
gyrB	GAAATGACCCGCCGTAA	456	52
	CTTGCCTTTCTTCACTTTGT		
blaKPC	GCTACACCTAGCTCCACCTTC	1050	52
	TCAGTGCTCTACAGAAAACC		
NDM-1	ATTAGCCGCTGCATTGAT	151	52
	CATGTCGAGATAGGAAGTG		
oqxA	CTCGGCGCGATGATGCT	393	52
	CCACTCTTCACGGGAGACGA		
oqxB	TTCTCCCCCGGCGGGAAGTAC	512	52
	CTCGGCCATTTTGGCGCGTA		
OXA	ACAGAAGCATGGCTCGAAAGT	190	52
	TTGCTGTGAATCCTGCACCA		
ParC	CTGAATGCCAGCGCCAAAT	567	52
	GCGCATACGCACTGAAC		
qepA	GCAGGTCCAGCAGCGGGTAG	218	52
	CTTCCTGCCCGAGTATCGTG		
qnrA	ATTTCTCACGCCAGGATTTG	516	52
	GATCGGCAAAGGTTAGGTCA		
qnrB	GATCGTGAAAGCCAGAAAGG	476	52
	ATGAGCAACGATGCCTGGTA		
qnrC	GGGTTGTACATTTATTGAATC	447	52
	TCCACTTTACGAGGTTCT		
qnrD	CGAGATCAATTTACGGGGAATA	582	52
	AACAAGCTGAAGCGCCTG		
qnrS	ACGACATTCGTCAACTGCAA	417	52
	TAAATTGGCACCCTGTAGGC		
Su12	GATGGCATTCCCGTCTC	577	52
	TTCTTGCGGTTTCTTTCAGC		

TABLE 2
Antibiotic susceptibility of *Klebsiella pneumoniae* isolated from N. atra

Antimicrobial agents	Isolate	Antimicrobial agents	Isolate
AM	R	CIP	R
PIP	R	LVF	R
CZ	R	LMF	R
CTX	S	OFL	R
CFX	S	NOR	R
AZT	S	NAL	R
GM	R	C	R
TM	S	FT	R
AN	S	SMX	R
K	S	SXT	R
NET	S	AMX/CA	I
S	S	AM/SU	R
TE	R	PIP/TA	I
DO	R		

S: Susceptible; R: Resistant; I: Intermediate

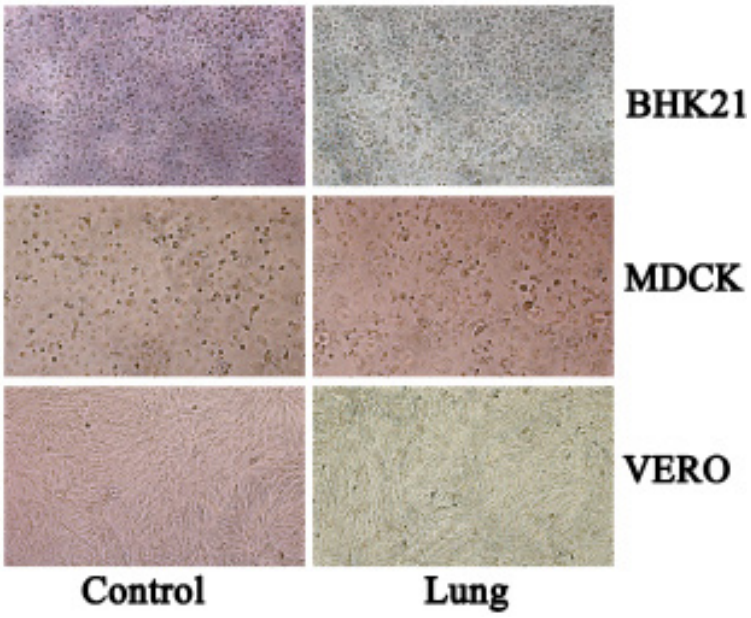

FIGURE 1. Lack of cytopathogenic effect of filtrates of lung homogenates of *Naja naja* on BHK21, MDCK and VERO cells.

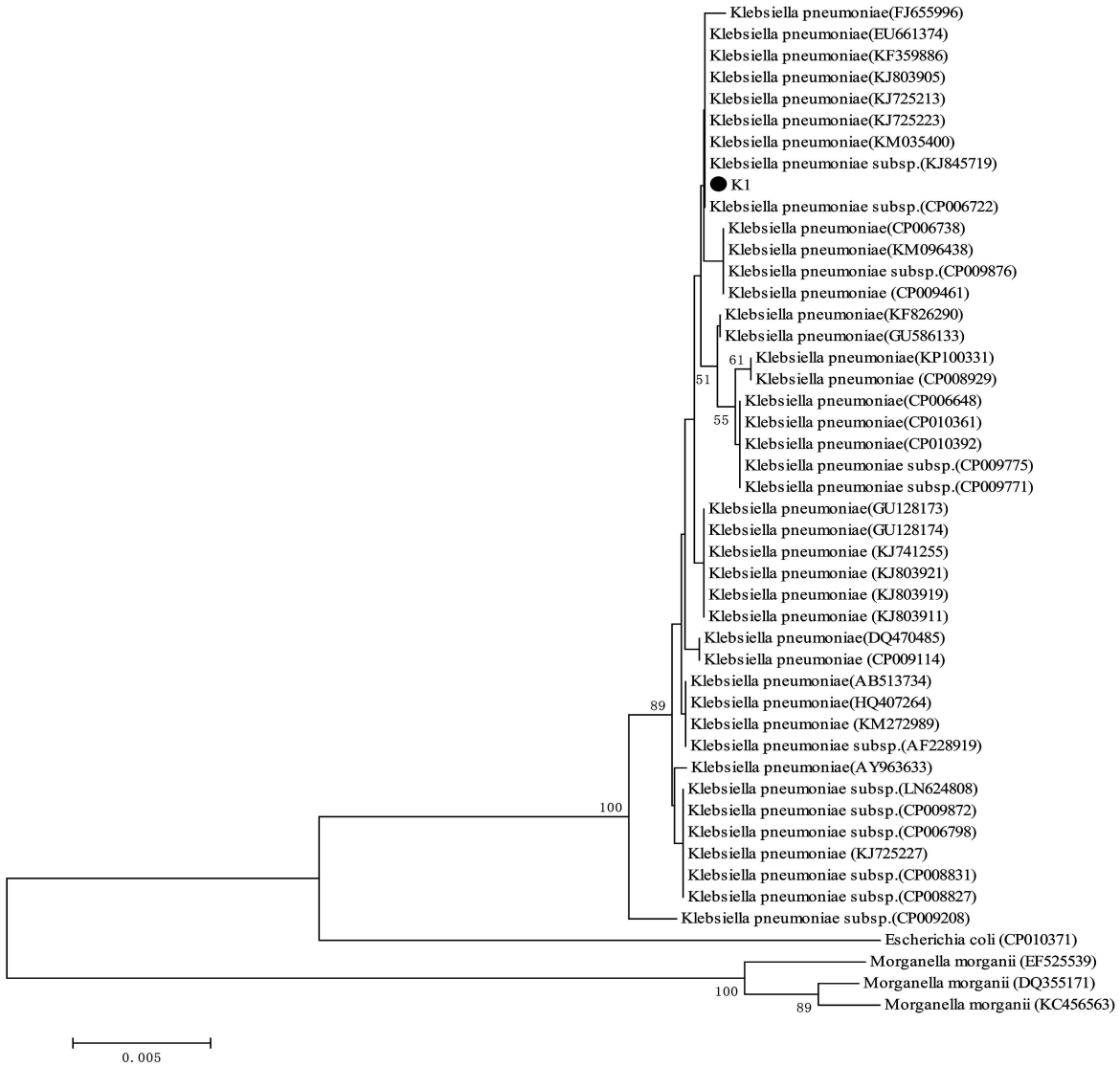

FIGURE 2. Phylogenetic tree including the 16SrDNA segment of *Klebsiella pneumoniae* isolated from *Naja atra*. The phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates. K1 indicates the sequence found in isolates of *Klebsiella pneumoniae* in this study.

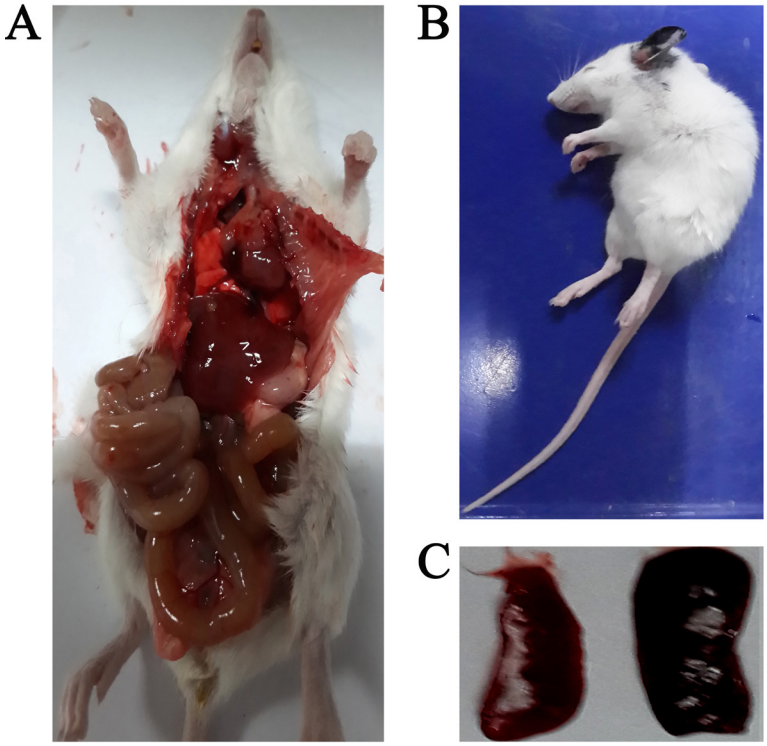

FIGURE 3. Effects of a *Klebsiella pneumoniae* isolate inoculated to BALB/C mice experiments of the *K. pneumoniae.* (A), severe intestinal edema; (B), mouse showing quivering, weight loss and expiratory dyspnea; (C) spleens from a control (left) and an inoculated mouse (right). Notice spleen enlargement and interstitial bleeding in the inoculated mouse.

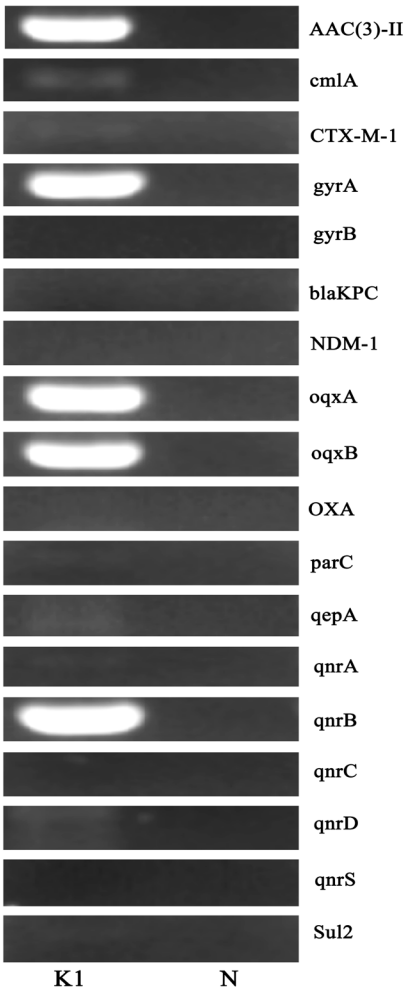

FIGURE 4. Detection of resistance genes in a *Klebsiella pneumoniae* isolate. Gel electrophoresis of *AAC(3)-II, cmlA, CTX-M-1, gyrA, gyrB, blaKPC, NDM-1, oqxA, oqxB, OXA, parC, qepA, qnrA, qnrB, qnrC, qnrD, qnrS,* and *Sul2* resistance genes amplicons; K1: *Klebsiella pneumoniae* isolate; N: negative control.

Antimicrobial susceptibility testing of K. pneumonia

Our three *K. pneumoniae* isolates from the lung of *N. atra* had the same resistance spectrum. They were resistant to ampicillin (AM), piperacillin (PIP), cefazolin (CZ), gentamicin (GM), tetracycline (TE), doxycycline (DO), ciprofloxacin (CIP), levofloxacin (LVF), lomefloxacin (LMF), ofloxacin (OFL), norfloxacin (NOR), nalidixic acid (NAL), chloramphenicol (C), nitrofurantoin (FT), sulfamethoxazole (SMX), sulfamethoxazole/trimethoprim (SXT), and ampicillin/sulbactam (AM/SU). In addition, the three isolates were susceptible to cefotaxime (CTX), cefixime (CFX), aztreonam (AZT), bramycin (TM), amikacin (AN), kanamycin (K), netilmicin (NET), and streptomycin (S). The resistance profiles of the *K. pneumoniae* isolates is presented in Tab. 2.

Detection of resistance genes

Our study showed that *AAC(3)-II*, *gyrA*, *oqxA*, *oqxB*, and *qnrB* coexisted in the *K. pneumoniae* isolates. However, *cmlA*, *CTX-M-1*, *gyrB*, *blaKPC*, *NDM-1*, *OXA*, *parC*, *qepA*, *qnrA*, *qnrC*, *qnrD*, *qnrS*, *oqxA*, and *Sul2* resistance genes were not amplified (Fig. 4).

Discussion

We were initially interested on the microbial associations of *N. atra*, because this snake has both food and medical significance in China, where the consumption of snake meat has been continuously increasing (Liu *et al.*, 2005; Wang, 2009; Yan *et al.*, 2013; Zhang *et al.*, 2016). In addition, because this snake is appreciated for its use in traditional Chinese medicine, in which its venom is used against pain, rheumatic disorders and cancer (Fu *et al.*, 2017; Huang and Hu, 2017) and the snake gallbladder is used for the treatment of respiratory diseases (Huang and Li, 2010; Yan, 2013; Zhong *et al.*, 2017).

Also, in order to meet the demand for snake products, the breeding of snakes is becoming increasingly common in China. However, severe viral and bacterial diseases may affect snake breeding (Allender *et al.*, 2006; Allender *et al.*, 2018;

Heldstab and Bestetti, 1984; Hetzel *et al.*, 2013; Jacobson *et al.*, 1980; Schumacher *et al.*, 1994; Uccellini *et al.*, 2014; Vieler *et al.*, 1994). At present, we do not know whether the *N. atra* individuals used in the current study had acquired *K. pneumoniae* from the environment or from other animals, or whether *N. atra* may act as a reservoir for this pathogen (Dantur *et al.*, 2018; Du *et al.*, 2014; Ghafur *et al.*, 2018; Podder *et al.*, 2014).

No evidence for viruses was found in our study, in which no cytopathogenic effects of filtrates of lung homogenates were observed, either on BHK-21, MDCK, or VERO cells in culture, which suggests that there were no viruses in the samples.

However, we did find *K. pneumoniae* in lung samples of *N. atra*, an enterobacterium that is a major pathogen in humans and animals (Guo *et al.*, 2015; Wang *et al.*, 2017). Recent studies in animals have found *K. pneumoniae* can infect African green monkeys, and hypermucoid phenotype of *K. pneumoniae* appears to be an important virulence factor that promotes escape from innate immune defenses (Cox *et al.*, 2015). Also, studies have shown that *K. pneumoniae* is commonly associated with dog urinary tract infections, primate multisystemic abscesses, mare metritis, and ruminant mastitis (Kikuchi *et al.*, 1987; Lin *et al.*, 2011; Soto *et al.*, 2012).

We are here reporting, for the first time, the occurrence of the resistance genes *AAC(3)-II*, *gyrA*, *oqxA*, *oqxB*, and *qnrB* in *K. pneumoniae* isolates from *N. atra*, as well as the absence of *cmlA*, *CTX-M-1*, *gyrB*, *blaKPC*, *NDM-1*, *OXA*, *parC*, *qepA*, *qnrA*, *qnrC*, *qnrD*, *qnrS*, *oqxA*, and *Sul2* genes.

Antimicrobial susceptibility testing of our *K. pneumoniae* isolates showed they were resistant to SMX and SXT, but no expression of *Sul2* was found in this study, a gene which is generally associated with resistance to sulfonamide drugs (e.g. Wang *et al.*, 2014). This finding indicates that other mechanisms of sulfonamide resistance may be present.

Regarding drug resistance genes in China, *blaNDM-1*, *blaCTX-M-15* and *blaCTX-M-14* were present in *K. pneumoniae* isolates from a neonatal ward of Shandong Province, China (Jin *et al.*, 2015). Our failure to find the resistance genes *KPC*, *NDM*, and *OXA* in the isolates from *N. atra* may indicate that carbapenem antibiotics may still be effective in the multidrug resistant *K. pneumoniae* strains isolated from *N. atra*.

Also in this study, *K. pneumoniae* isolates showed a relatively high resistance to cefazolin, a first generation cephalosporin, while being still sensitive to cefotaxime, a third generation cephalosporin. This illustrates that the widespread use of some antibiotics (such as cephalosporins) may lead to the emergence of resistant strains eventually entering the human food chain. Another example may be the detection of quinolone resistance in isolates of the current study, together with the expression of the resistance genes *oqxA*, *oqxB* and *qnr B*. In addition, our study showed *GyrA* gene is present in *K. pneumonia*, but *GyrB* does not appear. DNA gyrase and topoisomerase IV mutations are the main mechanisms of quinolone resistance (Robicsek *et al.*, 2006), however these findings imply that the integrity of DNA gyrase may not be the key to the survival of *K. pneumoniae*, and this will need further study.

Regarding carbapenem, which is the most effective antimicrobial compound for treatment of Gram-negative bacteria, its widespread use has accelerated the growth of carbapenem-resistant strains (Jin *et al.*, 2015). For example, a recent study indicates carbapenemase-producing *K. pneumoniae* from clinical isolates possess the four most prevalent carbapenemase types in Germany (*KPC-2, KPC-3, OXA-48, NDM-1*) (Becker *et al.*, 2018). In addition, coproduction of OXA-48 and NDM-1 was observed following clonal diversity and genetic profiling of antibiotic resistance among multidrug/carbapenem-resistant *K. pneumoniae* in Saudi Arabia (Zaman *et al.*, 2018).

In conclusion, our study first illustrated the resistance genes profile of *K. pneumoniae* that was isolated from *N. atra* in Northern China and revealed this case as a reminder that *K. pneumoniae* may be associated with zoonotic infections, and with reptiles in particular.

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