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Original Research

Isolation, identification and characterization of *Morganella morganii* from *Naja naja atra* in Beijing, China

H-F. Wang[1, 2], L-Y. Du[2,3], J. Luo[2], H-X. He[2*]

[1] Department of Environmental and Chemical Engineering, Yellow River Conservancy Technical Institute, Dong Jing Avenue, Kaifeng, People's Republic of China
[2] National Research Center for Wildlife-Borne Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beichen West Road, Chaoyang District, Beijing, People's Republic of China
[3] College of Life Science, Hebei Normal University, South Second Ring East Road, Shijiazhuang, People's Republic of China

Correspondence to: hehx@ioz.ac.cn

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Abstract: *Morganella morganii* is an important opportunistic human pathogen and belongs to the family of *Enterobacteriaceae*. Although it is widely distribution, it only be considered a rare cause of human infections. We report the isolate of *M. morganii* from *Naja naja atra* following infections of heart, lung and liver. Seven strains were confirmed using 16S rDNA amplified and sequences. Antimicrobial susceptibility testing of *M. morganii* isolates demonstrated ubiquitous resistance to ampicillin, amoxicillin/clavulanic acid, cefazolin, cephalothin, sulfamethoxazole/trimethoprim, sulfamethoxazole et al. However, *M. morganii* ubiquitous susceptible to piperacillin, ampicillin/sulbactam, piperacillin/tazobactam, cefixime et al. Further investigate display gyr B and Sul2 genes presence in all *M. morganii* isolates. AAC(3)-II was found in E2, E3 and E6 *M. morganii*. gyrA and qnrB expression in M3 and M6 *M. morganii*. This is the first description in *M. morganii* carrying AAC(3)-II, gyrB, gyrA, qnrB, and Sul2 genes from *Naja naja atra*, which suggests the increasing risk of pathogen transmission between humans and wildlife.

Key words: Antibiotics; Resistance genes; *Morganella Morganii*; *Naja naja atra*.

Introduction

Morganella morganii is a gram-negative bacteria commonly found in the environment and as normal flora in the intestinal tracts. The genus *Morganella* belongs to the family *Enterobacteriaceae*, contains only a single species, *M. morganii* (1-3). It is usual in the environment and can induce nosocomial outbreaks and infections in immunocompromised patients (4). Study display *M. morganii* mutants accompany with AmpC expression are resistant to third-generation cephalosporins, monobactams, and cephamycin (5). *M. morganii* as an important nosocomial pathogen has been attributed a high mortality rate in some infections (6). Apart from *M. morganii* intrinsic resistance to colistin, it is naturally drug resistant to tigecycline, tetracyclines, macrolides, fosfomycin, b-lactams, nitrofurantoin and polymyxins (7, 8).

Although it is widely distributed, but it is often encountered in postoperative and other nosocomial settings, causes a variety of clinical infections such as sepsis, urinary tract infections, pneumonia, wound infections, skin and soft tissue, central nervous system infections, septic arthritis, meningitis, chorioamnionitis, neonatal sepsis, and peritonitis (9-15). Plasmid-mediated quinolone resistance (PMQR) mechanisms have been occured during the past decade, including Qnr proteins, QepA transporters, and the acetyltransferase AAC(60)- Ib-cr (16, 17). The qnrD gene was first described in a human clinical isolate of Salmonella enterica serovar Kentucky and three Salmonella enterica serovar Bovismordificans isolates from China (18). New Delhi metallo-b-lactamase (NDM-1) as a significantly resistant gene first described in Sweden from a patient who had previously been hospitalized in New Delhi, India and rapid dissemination worldwide (19). Artifical snake breeding as a traditional have existed for hundreds of years and there are many snake farms distribution in china. Snake was mainly used in diet and traditional chinese msdicine. However, few reports have addressed the characteristic of *M. morganii*. The aim of this study was to assess the clinical manifestations, antimicrobial susceptibilities and resistance genes pattern of *M. morganii* from *Naja naja atra*.

Materials and Methods

Samples collection and identification

The tissue samples were obtained from heart, liver and lung of *Naja naja atra* in Beijing, China. Sample were cultured on Mueller hinton agar medium used bacterial inoculation loop under aseptic condition at *37 °C* for 18 h. Bacterial colonies from each sample and polymerase chain reaction (PCR) amplification of 16S rDNA gene was performed with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R

Table 1. Resistance genes primer sequences, amplicon size and annealing temperature used in PCR assays.

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

(5'-GGTTACCTTGTTACGACTT-3') (20). BD Phoenix™ 100 (Maryland, USA) was used process biochemically test in order to identification of the bacterium.

Infection testing of homogenates

BHK21 (Baby Hamster Syrian Kidney), MDCK (Madin-Daby canine kidney), 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 heart, lung, and liver organs homogenates from *Naja naja atra* flowing 0.22μm filter were ino-culated on BHK-21, MDCK, and VERO cells for 5days, respectively.

Histopathological examination

Necropsies and tissue sampling of *Naja naja atra* were performed according to a standard protocol. After fixation in 10% neutral buffered formalin and embedding in paraffin, tissue sections were stained with hematoxylin and eosin for histological evaluation.

Phylogenetic analyses

Sequences compared to the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/) . Alignments of clones and reference sequences were created with Clustal X. Phylogenetic analyses were performed using the neighbor-joining method with 1,000 bootstraps using MEGA 6 following the kimura 2-parameter model (21).

Antibiotics susceptibility testing

The antibiotics susceptibility analysis of isolates to different antimicrobial drug were determined using disk diffusion test according to CLSI (Clinical and Laboratory Standards Institute) guidelines recommendation and manufacturers' instructions.

Detection of resistance genes

PCR detection and gene identification were carried out for AAC(3)-II, cmlA, CTX-M-l, gyrA, gyrB, blaKPC, NDM-1, oqxA, oqxB, OXA, parC, qepA, qnrA, qnrB, qnrC, qnrD, qnrS and Sul2 genes (22-25). Sequences were compared to NCBI database. These primes were detected in present study seen in Table 1.

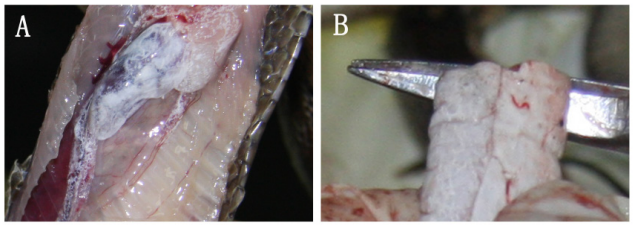

Figure 1. The severe fibrous exudate of heart and lung from *Naja naja atra.* The fibrous exudate of heart (A) and lung (B) from disease *Naja naja atra*.

Results

Case description

In October 2014, a *Naja naja atra* presented to the National Research Center for Wildlife Born Diseases (NCWBD) at the Institute of Zoology (IOZ), Chinese Academy of Sciences (CAS) from a snake farm of Beijing outskirts. The snake displayed depression, inappetence, and labored respiration before died. Snake was placed in the disc of the dissection and observe the surface of the snake, used sterile scissors from the anus to the head, ensure the kidneys, spleen, liver and other organs without lesions. Under sterile environment, heart, liver, kidney, lung and other organs were removed from snake. The surface of the internal organs was sterilized by flame, and the organs was cut with sterile scissors. Samples were got at the lesion site use inoculation ring and seeded on the nutrient agar plate by plate scribing method (Figure 1).

Identification of *M. morganii*

Samples were cultured on Mueller hinton-agar medium at *37 °C* for 18 h. Bacterial colonies from each sample were firstly gram-stained and PCR products were detected by 1 % agarose gel electrophoresis, the results showed that the amplified products were 1500 bp in length with good specificity, which consistent with the expected fragment (Figure 2). Amplify Products of 16S rDNA gene of bacterial colonis were purified for DNA sequencing. Results suggested that *M. morganii* (M1-7) as the only pathogen microbe widespread in visceral organs (Table 2). In addition, the isolates also were confirmed as *M. morganii* with used the BD Phoenix™ 100 (Maryland, USA).

Pathological changes of visceral organs

Naja naja atra were necropsied in order to observed pathological change. Typically, obvious thick white cellulose exudate covered in surface of lung, heart, and liver tissues, the lungs showed edema, the heart was enlarged and bleeding, the liver showed diffuse bleeding. Histopathological shown pulmonary edema, diffuse bleeding, and alveolar wall thickening in the lung, and myocardial display hyperplasia and fibrin exudation in heart slice, further observed display that congestion, edema, necrosis and lymphocytes increased in liver cells (Figure 3).

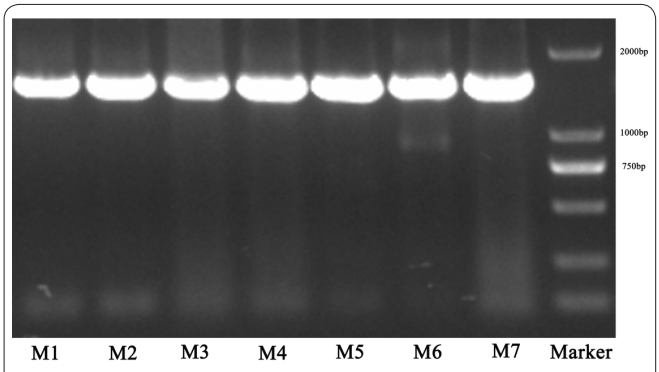

Figure 2. The 16S rDNA PCR result of seven bacterial colonies from *Naja naja atra.*

Table 2. The tissues sites of *M. morganii* colonies from *Naja naja atra*.

Name	Species	Sample source
M1	Naja naja atra	liver
M2	Naja naja atra	lung
M3	Naja naja atra	heart
M4	Naja naja atra	liver
M5	Naja naja atra	heart
M6	Naja naja atra	lung
M7	Naja naja atra	Liver

Cells with normal morphology

In order to determine whether the presence of viral infection, supernatant of heart, lung, and liver organs homogenates were cultured with BHK-21, MDCK, and Vero cells from *Naja naja atra* for 5days, respectively. Our study showed cell morphology did not occur significantly change compared to control (Figure 4).

Phylogenetic analysis

The 16S rDNA gene sequences showed a closer si-

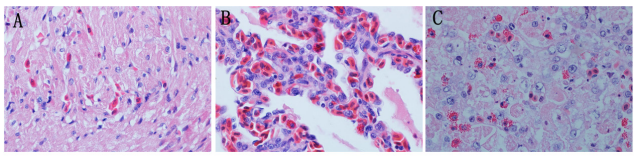

Figure 3. Histopathological analysis of heart, lung and liver of disease *Naja naja atra.* Myocardial hyperplasia and fibrin exudation in heart slice HE (A). Pulmonary haemorrhage, edema and alveolar wall thickening in lung slice HE (B). Congestion, edema, necrosis and lymphocytes increased in liver slice HE (C). Images at magnifications of 600×are shown.

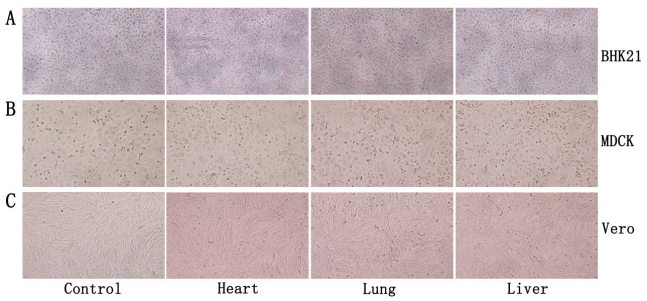

Figure 4. Homogenates of heart, lung, and liver of *Naja naja atra* were incubated with BHK21, MDCK and Vero cells. The homogenates of heart, lung and liver from disease snake were co-cultured with BHK21 cells, not found cytopathic compared to the negative control (A). The homogenates of heart, lung and liver from disease snake were co-cultured with MDCK cells, not found cytopathic compared to the negative control (B). The homogenates of heart, lung and liver from disease snake were co-cultured with Vero cells, not found cytopathic compared to the negative control (C).

Table 3. Antibiotic susceptibility of *Morganella morganii* were isolated from *Naja naja atra* in Beijing, china.

Antimicrobial agents	Strains						
	M1	M2	M3	M4	M5	M6	M7
AM	R	R	R	I	R	R	R
PIP	S	S	I	S	S	S	S
CZ	R	R	R	R	R	R	R
CF	R	R	R	R	R	R	R
CTX	S	R	I	R	I	R	I
CFX	S	S	S	S	I	S	S
AZT	S	S	S	S	S	S	I
GM	S	R	R	R	R	R	I
TM	S	S	S	I	R	R	S
AN	S	S	S	S	S	S	S
K	I	I	I	R	I	R	R
NET	S	S	S	S	S	S	S
S	S	R	R	R	R	R	R
TE	I	I	I	R	I	I	R
DO	R	I	R	R	I	R	S
CIP	S	S	I	I	S	R	S
LVF	S	S	S	S	S	R	S
LMF	I	I	R	R	R	R	S
OFL	S	S	S	I	S	I	S
NOR	S	S	S	S	S	S	S
NAL	S	I	I	I	I	I	I
SMX	R	R	R	R	R	R	R
C	R	R	R	R	R	R	R
FT	S	S	R	R	R	S	S
AM/SU	S	S	S	S	S	S	S
PIP/TA	S	S	S	S	S	S	S
SXT	R	R	R	R	R	R	R
AMX/CA	R	R	R	R	R	R	R

S, Susceptible; R,Resistant; I, Intermediate.

milarity to KJ794191 and KF751866 have the highest nucleotide identity and topological structural (Figure 5). KJ794191 illustrated come from southwest university of china through direct submission to NCBI (National Center of Biology and Information). KF751866 was isolated from seafood processing wastewater and spoilaged squid from Thailand. Above results suggested that *M. morganii* strains from *Naja naja atra* may be related to aquatic ecosystems.

Antimicrobial susceptibility testing of *M. morganii*

Our study shown *M. morganii* isolates ubiquitous resistance to ampicillin (AM), amoxicillin/clavulanic acid (AMX/CA), cefazolin (CZ), cephalothin (CF), gentamicin (GM), sulfamethoxazole/trimethoprim (SXT), sulfamethoxazole (SMX), and chloramphenicol (C). In addition, *M. morganii* ubiquitous susceptible to piperacillin (PIP), ampicillin/sulbactam (AM/SU), piperacillin/tazobactam (PIP/TA), cefixime (CFX), aztreonam (AZT), amikacin (AN), netilmicin (NET), norfloxacin (NOR). *M. morganii* strains existence different level of resistance tobramycin (TM), doxycycline (DO), ciprofloxacin (CIP), levofloxacin (LVF), lomefloxacin (LMF) and nitrofurantoin (FT). The resistance profiles of the seven strains are presented in Table 3.

Detection of resistance genes

According to antibiotic susceptibility of *M. morganii* from *Naja naja atra*. Further investigate display gyr B and Sul2 genes presence in all *M. morganii* isolates. AAC(3)-II was found in E2, E3 and E6 *M. morganii*. gyrA and qnrB expression in M3 and M6 *M. morganii*. OqxB shown a weak PCR band in M3 and M6 isolates, CTX-M-1 shown a weak PCR band in M7 isolated. cmlA, blaKPC, NDM-1, oqxA, OXA, parC, qepA, qnrA, qnrC, qnrD, and qnrS resisitance genes not found

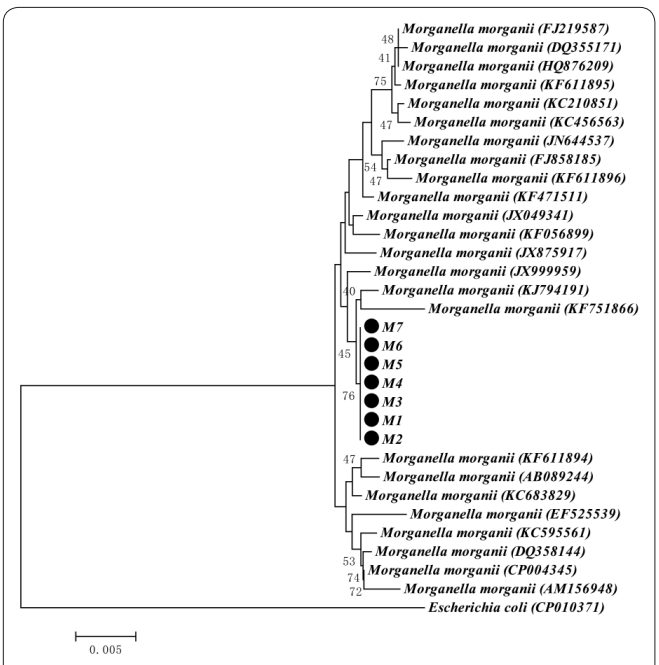

Figure 5. Phylogenetic tree based on 16SrDNA segment of *Morganella morganii* from *Naja atra* in Beijing, china. Phylogenetic tree based on partial 16S rDNA gene sequences of *M. morganii*. The phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates. GenBank accession numbers for the sequences used in the study are shown in parentheses. A black circle indicates sequence generated in this study.

amplify bands (Figure 6). These results suggests different resistance genes profile were found from several *M. morganii* isolates. This is the first description in *M. morganii* carrying AAC(3)-II, gyrB, gyrA, qnrB, and Sul2 genes was isolated from *Naja naja atra*. However, gyrB existence in all *M. morganii* isolates implied gyrA may not be necessary for normal survive of *M. morganii*.

Discussion

Morganella morganii is an important opportunistic human pathogen, it rarely causes disease in healthy individuals but occured mainly in the hospital setting (26-32). Our study confirmed that drug resistant *M. morganii* was responsible for the *Naja naja atra* disease. *M. morganii* has a low pathogenicity, but compromised patients can develop diarrhea, wound infections, urinary tract infections, bacteremia, and sepsis (33). We report *M. morganii* were found in heart, lung and liver following inflammation of internal organs from *Naja atra* in north China. Roels S et al. report *M morganii* was isolated from a domestic rabbit with bronchopneumonia

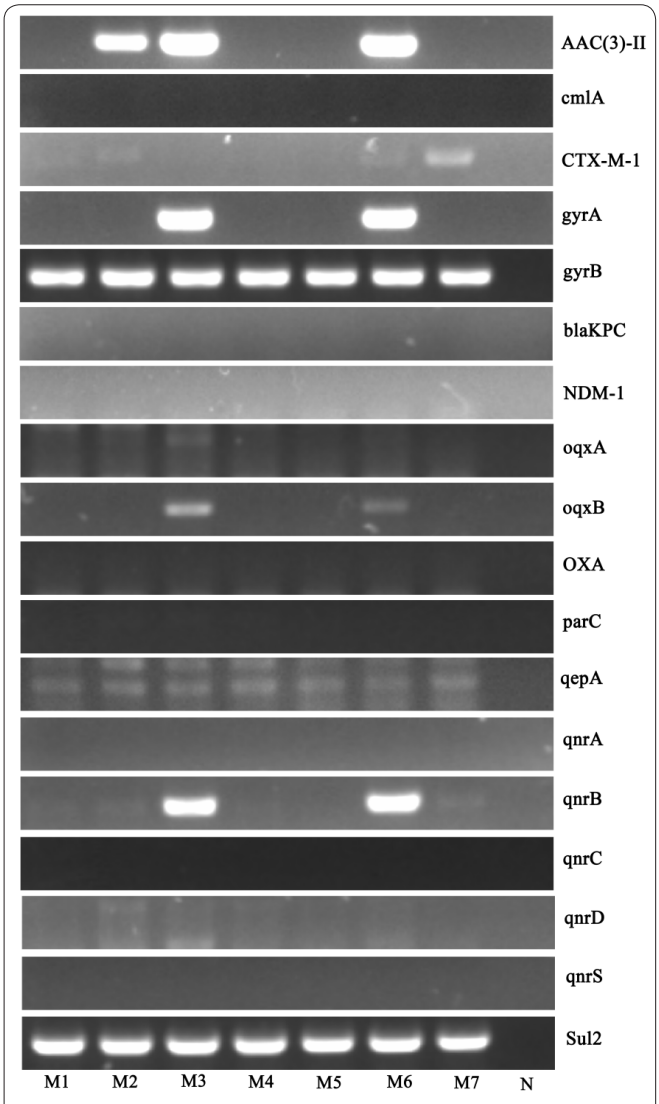

Figure 6. Resistance genes analysis of the *Morganella morganii* from *Naja naja atra*. Gel electrophoresis of AAC(3)-II, cmlA, CTX-M-1, gyrA, gyrB, blaKPC, NDM-1, oqxA, qepA, and OXA amplicon products; M1, M4 and M7 were isolated from liver; M2 and M6 were isolated from lung; M3 and M5 were isolated from heart; N:negative control.

(34). Plasmid-mediated quinolone resistance has been described in many *Enterobacteriaceae* clinical isolates worldwide. Zalas-Wiecek P et al. found *M morganii* strains were susceptible to carbapenems but decrease susceptible to piperacillin and chloramphenicol. Study shown *M. morganii* chromosomal existence A class 1 integron with different gene cassettes (dfrA1, orfC and aadB) (35). *M. morganii* resistance to β-lactam antibiotics is usually mediated by the β- lactamases belonging to the AmpC β-lactamase family through chromosomal encoded (36, 37). Diene SM et al. report five CTX-M-15-producing *Morganella morganii* were isolates from Hôpital Principal de Dakar, Senegal (38). Our results display ampicillin, amoxicillin, and "first-generation" cephalosporinsare ineffective to *M.morganii*. In contrast, the *M. morganii* were susceptible to advanced cephalosporins (CFX) and penicillins in this study. In addition, phages have recently been suggested as an alternative antibacterial agent to counteract the emergence of antibiotic-resistant *M. morganii* for treatment of infectious disease or food decontamination (39, 40). Study shown *M. morganii* isolates were resistant to penicillins, aztreonam, and ciprofloxacin, however, were susceptible to amikacin from human (41). Aminoglycoside resistance toward *M. morganii* by various combinations of enzymes, ANT(2)-I confers resistance to gentamicin, tobramycin, and kanamycin is the most commonly modifying enzyme (42). Our research display that *M. morganii* difference resistance toward gentamicin, tobramycin, and kanamycin, these result demonstration *M. morganii* can provides distinguish resistance mechanism. Generally, *M. morganii* infections are due to post-operative wound and urinary tract infection (43). No study have described the mechanism of antibiotic resistance of *M. morganii* from *Naja naja atra*.

Previous report shows *M. morganii* can was detected from some aquatic products such as gill and skin of mackerel, sardine, and albacore. Meanwhile, the surface of conveyer belts and plastic totes contacted with mackerel and sardine is contaminated during processing although no *M. morganii* was found in the processing plant before processing (44). Our study reveals *M. morganii* were found in the internal organs of the snake, whether venom of snakes exist *M. morganii* need further research. *M. morganii* were separated from cobra guts, because *M. morganii* widely exists in nature environmentl especially in the hospital environment, that may indicate *M. morganii* from human to snake, however, our study shows more like a secondary infection, but *M. morganii* resistance has undergone significant changes.

In conclusion, our result shown *M. morganii* carried different resistance genes induce to distinctly antibiotics susceptibility increasing the difficult of treatment. Again stressed the importance of antibiotic susceptibility testing to prevention and control of antibiotic abuse to wildlife as well as reveal this case to create the awareness that *M. morganii* possible have a link between humans and wildlife.

Acknowledgements

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