Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Detection of drug-resistant *Kluyvera intermedia* from *Zoacys dhumnades*

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ARTICLE INFO

Original paper

Article history:
Received: August 27, 2022
Accepted: September21, 2022
Published: September 30, 2022

Keywords:

Zoacys dhumnades, Kluyvera intermedia; isolation, resistance

ABSTRACT

Zoacys dhumnades is native to china and has important economic and medicinal value, but the pathogenic microorganisms have been reported rarely. *Kluyvera intermedia* is usually considered a commensal. In this study, *Kluyvera intermedia* was first isolated from *Zoacys dhumnades* identical *by the 16SrDNA sequence, phylogenetic tree analysis, and biochemical tests. Cell infection experimental* did not find cell morphology change significantly compared to control with pathological organs homogenates from *Zoacys dhumnades*. *Antibiotic susceptibility shown Kluyvera intermedia* isolates were sensitive to 12 kinds of antibiotics and resistant to 8 kinds of antibiotics. *Resistant antibiotic genes screening* display gyrA, qnrB, and sul2 were found in *Kluyvera intermedia*. This is the first report of *Kluyvera intermedia* associated fatality with *Zoacys dhumnades* suggesting the need for continuous monitoring of nonpathogenic bacteria antimicrobial susceptibility from human, domestic animals and wildlife.

Doi: http://dx.doi.org/10.14715/cmb/2022.68.9.15

Introduction

Kluyvera spp belonging to the *Enterobacteriaceae* family, gram-negative micro-organisms, include *Kluyvera georgiana*, *Kluyvera cochleae*, *Kluyvera ascorbata* and *Kluyvera cryocrescens*, *Kluyvera cochleae* is a later synonym of *Kluyvera intermedia (1, 2)*. *Kluyvera intermedia* as uncommon pathogens, cause human and animal diseases very rare. Recent research shows that *Kluyvera intermediate* is relatively abundant in *Melophagus ovinus* was isolated from sheep in Tibet, China (3, 4). A case report illustrated low birth weight infant associated with the growth of *Kluyvera ascorbata blood culture means Kluyvera ascorbata sepsis (5).* Han JE, et al. first report zoonotic pathogen *Kluyvera ascorbata was isolated from the Egyptian fruit-bat* Rousettus aegyptiacus reveal *Kluyvera ascorbata may be as zoonosis pathogen spread among humans and animals (6, 7).* In addition, Kluyvera cryocrescens can be as a serious pathogen that causes preterm infants sepsis (8-10). Microbial insecticides play a important role for the control of insect pest. Some bacterial isolates identified as *Kluyvera intermedia* O-1, O-8, O-10 and S-3 were isolated from pine forest soil and disply different insecticidal effort towards the pest (11).

Zoacys dhumnades is unique to china, as well as has important economic and medicinal value (12-14). In this report, we describe *Kluyvera intermedia* associated with the death of *Zoacys dhumnades* and resistance genes gyrA, qnrB, and sul2 were found in *Kluyvera intermedia*. To our knowledge, this is the first description of *Kluyvera intermedia* isolated from *Zoacys dhumnades*. This study demonstrated the potential risk for zoonotic bacterial transmission by eating wild-caught snakes or herbal medicinal sterilization incomplete and will be important meaning in public health concern about the *Zoacys dhumnades* utility.

Materials and Methods

Samples collection and bacterial identification

Tissue samples were isolated from the liver and lungs of *Zoacys dhumnades* in Beijing, China. Mueller Hinton agar medium was used to process bacterially culturing through an inoculation loop under the aseptic condition at *37 °C* for 18 h. Bacterial colonies used polymerase chain reaction (PCR) amplification of 16S rDNA gene was performed with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R (5'-GGTTACCTTGTTACGACTT-3') (15). BD Phoenix™ 100 (Maryland, USA) was used implement biochemically test in order to further identification of the bacterium.

Phylogenetic analyses

Bacterial colony PCR sequences compared to the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/). Phylogenetic analyses were performed using the neighbor-joining method with 1,000 bootstraps using MEGA 6 following the kimura 2-parameter model (16).

Histopathological examination

Liver of *Zoacys dhumnades* following fixation in 10% neutral buffered formalin and embedding in paraffin, tissue sections were stained with hematoxylin and eosin for histological evaluation.

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Infection testing of homogenates

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.* The supernatant of organ homogenates from *Zoacys dhumnades* flowing 0.22μm filter was ino-culated on BHK-21, MDCK, and VERO cells for 5 days, respectively.

Animal experimental

Five BAL B/C mice in each group were inoculated intraperitoneally with 0.2 mL (1×10^8 colony-forming units [CFU]/mL) of pure bacterial culture per 10g body weight or phosphate buffer as control. 16SrDNA sequences and BD Phoenix™ 100 were used for the bacterium identical. Ethical conduct in the care and use of experimental animals were supervised and the procedures were approved by the ethics committee on animal experimentation of the Institute of Zoology, Chinese Academy of Sciences.

Antibiotics susceptibility testing

The antibiotic resistance elevated of bacteria isolates to the different antimicrobial drug were determined using a disk diffusion test according to CLSI (Clinical and Laboratory Standards Institute) guidelines recommendation and manufacturers' instructions.

Detection of bacterial resistance genes

Resistance 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 (17-21). Conditions for PCR were initial denaturation at 95°C for 5 min; 35 cycles at 95°C for 30 s, 52 °C for 30 s, and 72 °C for 40 s; and final incubation for 10 min at 72 °C. All sequences were compared to the NCBI database.

Results

Identification of bacterial

PCR amplified products were 1500 bp in length by 1 % agarose gel electrophoresis with good specificity. Amplify Products of the 16S rDNA gene were purified for DNA sequencing, indicating *Kluyvera* spp as the pathogen microbe. These isolates were confirmed as *Kluyvera intermedia* with used the BD Phoenix™ 100 .

Phylogenetic analysis

The 16S rDNA gene sequences showed a closer similarity to *Kluyvera intermedia* (KM222638.1) have the highest nucleotide identity and topological structure.

Pathological changes in the liver

Kluyvera intermedia was found in the liver and lung of *Zoacys dhumnades.* Further histopathology reveals congestion, edema, necrosis and lymphocyte increased significantly in the liver (Fig. 1).

Cells with normal morphology

The supernatant of liver and lung organ homogenates from *Zoacys dhumnades* were cultured with BHK-21, MDCK, and VERO cells for 5 days, respectively. Our study showed cell morphology did not significantly change compared to the control (Fig. 2).

Kluyvera intermedia was isolated from animal test

After 24h, the experimental group BALB/C mice manifested as apathetic, losing appetite and abdominal breathing. All mice died within 48 hr after inoculation, while the control mice appeared normal. On necropsy, intestinal edema, heart hemorrhage, spleen and left lung severe bleeding were seen in dead mice. *Kluyvera intermedia* was isolated from BALB/C mice following confirmation by BD Phoenix™ 100 and 16SrDNA sequences.

Antimicrobial susceptibility testing of the isolates

Our results showed *Kluyvera intermedia* were sensitive to 12 kinds of antibiotics, including Aztreonam, Gentamicin, Tobramycin, Amikacin, Kanamycin, Netilmicin, Levofloxacin, Ofloxacin, Norfloxacin, and Nitrofurantoin, Ampicillin/Sulbactam, Piperacillin/Tazobactam, and resistant to Ampicillin, Cephalothin, Cefixime, Tetracycline, Doxycycline, Sulfamethoxazole/Trimethoprim, Sulfamethoxazole, and Chloramphenicol in Table 1.

Detection of resistance genes

Our results indicate gyrA, qnrB, and sul2 had significant expression in D1, D2, and D3 *Kluyvera Intermedia* isolates, but CTX-M-1 only appears in D1 *Kluyvera intermedia* isolate in Fig 3.

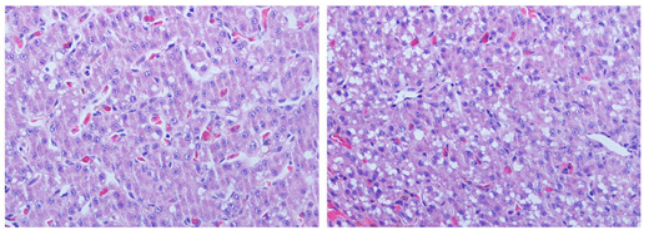

Figure 1. Histopathological analysis of liver of *Zoacys dhumnades.* Congestion, edema, necrosis and lymphocytes increased in liver slice HE. Images at magnifications of 400×are shown.

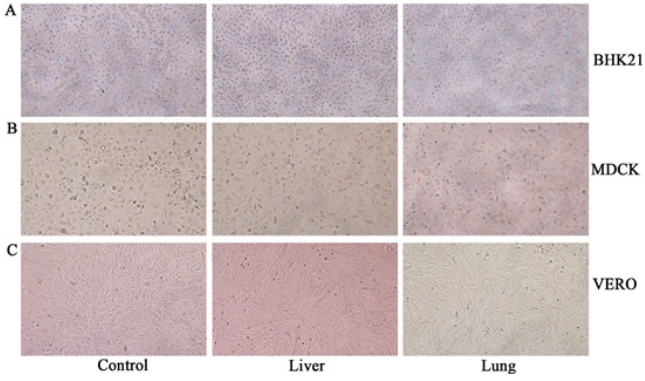

Figure 2. Homogenates of the liver and lung of *Zoacys dhumnades* were incubated with BHK21, MDCK and VERO cells. The homogenates of the liver and lung were co-cultured with BHK21 cells, and not found to be cytopathic compared to the negative control (A). The homogenates of the liver and lung were co-cultured with MDCK cells, not found cytopathic compared to the negative control (B). The homogenates of the liver and lung were co-cultured with VERO cells, not found cytopathic compared to the negative control (C).

Table 1. Antibiotic susceptibility of *kluyvera intermedia* was isolated *Zoacys dhumnade.*

Antimicrobial agents	Isolates		
	D1	D2	D3
Ampicillin(AM)	R	R	R
Piperacillin(PIP)	I	I	I
Cefazolin(CZ)	I	R	R
Cephalothin(CF)	R	R	R
Cefotaxime(CTX)	S	S	I
Cefixime(CFX)	R	R	R
Aztreonam(AZT)	S	S	S
Gentamicin(GM)	S	S	S
Tobramycin(TM)	S	S	S
Amikacin(AN)	S	S	S
Kanamycin(K)	S	S	S
Netilmicin(NET)	S	S	S
Streptomycin(S)	I	I	R
Tetracycline(TE)	R	R	R
Doxycycline(DO)	R	R	R
Ciprofloxacin(CIP)	S	I	I
Levofloxacin(LVF)	S	S	S
Lomefloxacin(LMF)	S	S	I
Ofloxacin(OFL)	S	S	S
Norfloxacin(NOR)	S	S	S
Nalidixic Acid(NAL)	I	S	I
Sulfamethoxazole/Trimethoprim (SXT)	R	R	R
Sulfamethoxazole (SMX)	R	R	R
Chloramphenicol(C)	R	R	R
Nitrofurantoin(FT)	S	S	S
Amoxicillin/Clavulanic Acid (AMX/CA)	I	S	S
Ampicillin/Sulbactam (AM/SU)	S	S	S
Piperacillin/Tazobactam (PIP/TA)	S	S	S

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

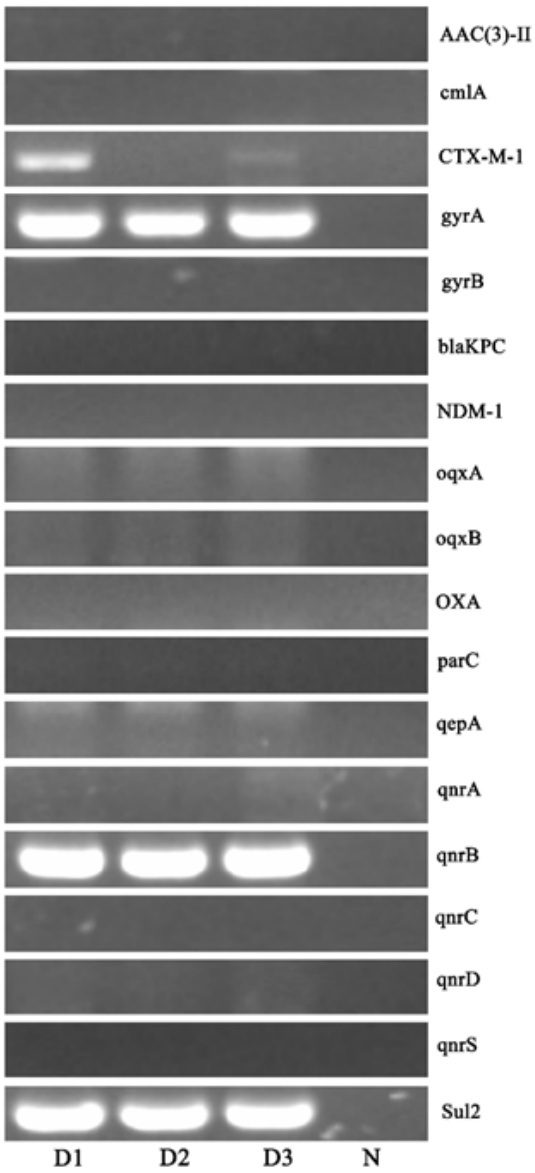

Figure 3. Resistance genes analysis of the *kluyvera intermedia* were isolated from *Zoacys dhumnades.* Gel electrophoresis of AAC(3)-II, cmlA, CTX-M-1, gyrA, gyrB, blaKPC, NDM-1, oqxA, oqxB, OXA, parC, qepA, qnrA, qnrB, qnrC, qnrD, qnrS, and Sul2 resistance genes amplicon products; D1 was isolated from the liver; D2 was isolated from the liver; D3 was isolated from lung; N: negative control.

Discussion

Antimicrobial resistance (AMR) is a great threat that human must face in the 21st century. *Kluyvera intermedia* is one of the most dominant isolates from municipal treated wastewater and showed significantly resistant against the antibiotics amoxicillin-clavulanic acid, cefuroxime, cefuroxime axetil and colistin, the study can help evaluate the harmful effects of wastewater reuse to regulate microbiological standards (22, 23). Generally, *Kluyvera* spp is a nonpathogenic and unusual human bacteria, but it has already been shown may actually as a reservoir of antimicrobial genes (24, 25). However, pathogenicity and resistance of *Kluyvera intermedia* report is very rare. Ribeiro VB et al. study indicates bla (GES-5) was detected in cabapenem-resistant *Kluyvera intermedia* isolates from a hospital environment (26, 27). Early studies have shown that *Kluyvera georgiana may be the progenitor of a subgroup of CTX-M-type beta-lactamases (24).* CTX-M-78 also was found in *Kluyvera georgiana* clinical isolate, suggests may be as the origin of the CTX-M-25 subgroup (28).

Moreover, previous research also displays bla_{KPC-2} gene was found in a *Kluyvera georgiana* isolate, and resistance related to the species of this genus remains rare (29). *In addition, Kluyvera ascorbata has a vital meaning due to its severity, such as severe sepsis, septic shock, and urinary tract infection (30, 31).* The study reveals the plasmid-borne quinolone resistance gene qnrA was found in *Kluyvera ascorbata that, illustrating resistance genes likely in vivo transfer through Kluyvera ascorbata (32).* Xu T, et al. study demonstrated a novel genotype of bla (KLUC) as close relatives with bla(KLUC-1) and bla(KLUC-2) was carried on the Kluyvera cryocrescens chromosome and Enterobacter cloacae plasmid (33).

In this case, our experimental indicate that Aztreonam, Gentamicin, Tobramycin, Amikacin, Kanamycin, Netilmicin, Levofloxacin, Ofloxacin, Norfloxacin, and Nitrofurantoin, Ampicillin/Sulbactam, Piperacillin/Tazobactam are suggested to be used in the treatment of *Kluyvera intermedia* infection. Due to gyrA, qnrB, and sul2 co-expression in *Kluyvera intermedia* that can explain resistance drugs such as Ampicillin, Cephalothin, Cefixime, Tetracycline,

Doxycycline, Sulfamethoxazole/Trimethoprim, and Sulfamethoxazole. In addition, these results may also demonstrate the relationship between humans and snakes pathogenic bacteria general cross-infection.

In summary, this study first revealed *K. intermedia* involved in *Zoacys dhumnades* death and gyrA, qnrB, and sul2 resistance genes were found in *Kluyvera intermedia.* Of course, we do not rule out *Zoacys dhumnades* may be infected via environmental exposure to *kluyvera intermedia* related to hospital waste and bacterial contamination of soil. Due to frequent human activities *kluyvera intermedia* possibly was transmitted from humans to *Zoacys dhumnades* suggesting increasing risk associated with wildlife, livestock, and humans, and multiple perspectives surveillance should be implemented.

Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Acknowledgments

This work was supported by the National Natural Sciences Foundation of China under grant number 31101806 and the science and technology development plan of Kaifeng in 2020 (2003048).

Conflict of Interest Disclosure

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work.

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