Morganella morganii as a Causative Agent of Disease in the Chinese Giant Salamander (Andrias davidianus)

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Abstract Morganella morganii strain 602-1 was isolated from a sick Chinese giant salamander (Andrias davidianus). The strain 602-1 was identified through its physiological and biochemical properties and 16S rDNA gene amplification and analyses. Pathogenicity was proven by experimental animal infection, and histopathological examination. The results showed that the amplified 16S rDNA sequence of strain 602-1 was 1455 bp, and showed 99% identity with M. morganii. In the host infection experiment, the mortality of Chinese giant salamanders was about 70%. Pathological changes occurred in the spleen, heart, liver, kidney and intestine. This study will help the prevention, understanding and cure for M. morganii infections in amphibians.

Keywords Morganella morganii, identification, pathogenicity, Chinese giant salamander

1. Introduction

Morganella morganii is a gram-negative rod, commonly found in the environment and in the intestinal tracts of human beings and animals (O’Hara et al., 2000; Falagas et al., 2006). M. morganii is considered to be an important intensive care unit pathogen and causes fatal massive hemolysis leading to death (Singla et al., 2010). The infection of skin and soft tissue by M. morganii is very common (Falagas et al., 2006). The most frequent cause of M. morganii bacteraemia is postoperative wound infection, and most infections occur in the patients who have received recent therapy with beta-lactam antibiotics. Other important epidemiological risk factors are the presence of diabetes mellitus, meningitis, septic arthritis, and so on (Falagas et al., 2006). M. morganii bacteraemia frequently occurs secondarily due to urinary tract or hepatobiliary tract infection, and is associated with a high mortality, especially for those not receiving appropriate antibiotic therapy (Lee and Liu, 2006). M. morganii is a rare perinatal pathogen, causing severe disease in premature infants, in association with maternal chorioamnionitis and premature rupture of the membranes (Ovalle et al., 2009). Presently, M. morganii has been isolated from chicken, domestic rabbit, human beings, and other animals (Chen et al., 2012; Zhao et al., 2012; Roels et al., 2007; Singla et al., 2010). Some studies on the isolation and virulence of M. morganii in human beings and some animals have been done, but few reports exist regarding amphibian infections caused by this microorganism (Chang et al., 2011).

The Chinese giant salamander (Andrias davidianus) belongs to Cryptobranchidae of Caudata. The Chinese giant salamander is listed as critically endangered animal by IUCN (2007), and is included in Appendix I of CITES. By far, the research on A. davidianus mainly focus on its artificial culture reproduction and genetics, only few on diseases of this animal (Yang et al., 2010; Zhang et al., 2006; Guo et al., 2012; Dong et al., 2011; Li et al., 2008). In this paper, a M. morganii strain was isolated from a sick A. davidianus and was diagnosed to species using 16S rDNA sequence analyses. Furthermore, its pathogenicity was explored in detail. To our knowledge,
it is the first report describing *M. morganii* infection in *A. davidianus*. It will benefit the identification and treatment of bacterial infection in *A. davidianus*.

2. Materials and Methods

2.1 Ethics statement The artificially cultivated Chinese giant salamanders can be legally traded and used in experiment according to Aquatic Wildlife Use Concession of China, which were also reported in some published articles (Geng et al., 2011; Ao et al., 2010; Wang et al., 2010). According to Koch’s postulates, the artificial infection experiment is an essential part to establish an infectious agent as the cause of a particular disease. All experiments involving live Chinese giant salamander were approved by the Animal Ethics Committee at Southwest University for Nationalities.

2.2 Sample collection and bacterial isolates The sick Chinese giant salamander was 2.46 kg in weight, and 71 cm in length. It stopped foraging and mobilization, then died a week later in Chengdu Aquarium. There were some small skin ulcers on the dorsal and ventral body surface. Samples from heart, liver and spleen were immediately collected and inoculated on blood plates. Next, the samples were subcultured onto general nutrition agar plates and incubated at 28 ºC for 18 h under aerobic conditions. Single colonies of bacterial strains were picked and cultured several times, on nutrient agar plates. Biochemical characterization of the strain was performed using the API 20E system.

2.3 16S rDNA gene amplification Bacterial DNA was extracted using genomic extraction kit of bacterium (Axygen, China) in accordance with the manufacturer’s protocol. The quality of DNA was evaluated on 0.8% agarose gel. DNA concentrations were determined in duplicate using a spectrophotometer. Polymerase chain reaction (PCR) reactions were employed to amplify the partial 16S rDNA gene, with the universal primers 27F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1492R (5’-ACG GCT ACC TTG TTA CGA CTT-3’). The reaction mixture (50 µl) contained 23 µl of rtaq Mix (TaKaRa, Japan), 21 µl of ddH2O, 2 µl of bacterial DNA, and 2 µl of each primer. The amplification steps included 94 ºC for 4 min, 94 ºC for 30 s, 50 ºC for 30 s, 72 ºC for 4 min (35 cycles). The PCR product was sequenced with the 16S rDNA primers by ABI PRISM 3730 automated sequencer.

2.4 Bioinformatics analysis DNASTAR Lasergene 6 was used to edit and assemble DNA sequences. Searches for homologous DNA sequences in GenBank were performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were generated using ClustalX program. Phylogenetic analyses were performed by neighbor-joining method with MEGA v3.1 Software. The reliability of the neighbor joining tree was estimated by bootstrap analysis with 1000 replicates.

2.5 Experimental animal infection Six healthy Chinese giant salamanders were artificially cultivated in Bazhong city, Sichuan province and used in experimental infection (mean weight, 135 ± 10 g). Experimental animals infections were conducted by injection as follows: three Chinese giant salamanders were injected in leg muscle with 0.5 ml of bacterial suspension (3 × 10^8 cfu/ml). In all cases, the controls were inoculated with the same dosage of PBS (pH 7.4) under the same conditions. The experimental animals were kept and observed for a week. Samples were taken immediately from the heart and the spleen of dead animals. Bacteriological analyses of dead animals were carried out in all the cases. In case the strain used for inoculation was isolated in pure culture, death could be considered caused by inoculated bacteria.

2.6 Histopathological examination The tissue samples, including major viscera (liver, lung, heart, kidney, spleen, stomach and intestine) were used to study the histopathological effects of *M. morganii*. The samples were collected from sick Chinese giant salamander and fixed in 10% buffered formalin and subsequently were dehydrated with a graded alcohol-xylene series. Dehydrated samples were embedded in toluene-paraffin, cut into 5 µm sections, and stained with hematoxylin-eosin. Changes in organizational structure were examined and visualized using a light microscope.

3. Results

3.1 Bacterial isolates After incubation at 28 ºC, a pure culture was identified and named strain 602-1. On nutrient agar, this strain appeared as non-hemolytic, convex colonies, 2–3 mm in diameter. The amplification steps included 94 ºC for 4 min, 94 ºC for 30 s, 50 ºC for 30 s, 72 ºC for 4 min (35 cycles). The PCR product was sequenced with the 16S rDNA primers by ABI PRISM 3730 automated sequencer.

3.2 Analysis of 16S rDNA gene The length of this 16S rDNA sequence was 1455 bp, and showed 99% identity with *M. morganii* (AJ301681.1). This
sequence was submitted to GenBank with an accession number of JX049341. A phylogenetic analysis of 16S rDNA sequences was performed with 14 sequences, including strain 602-1 isolated in this study and 13 sequences deposited in the GenBank database. Analyzed sequences were divided into two clusters, *Morganella* and *Citrobacter*. Strain 602-1 belonged to the group of *Morganella*, and grouped together with *Morganella morganii* (HQ169126.1), as the closest neighbour (Figure 1). Strain 602-1 was confirmed to be *M. morganii* on the basis of sequence analyses.

### 3.3 Experimental animal infection

In this artificial infection experiment, after a week of post infection experimental the Chinese giant salamanders exhibited a variety of symptoms and lesions, which were in agreement accorded with those described above. Two of the Chinese giant salamanders died within a week, and the mortality was about 70%. During this week, all the controls were alive and had no clinical symptoms. By means of isolation and identification, the *M. morganii* strain was obtained again from all moribund and dead Chinese giant salamanders after the infection experiment.

### 3.4 Histopathological analysis

Light microscopic examination showed the lesions were mainly observed in the heart, liver, kidney, intestine, spleen and stomach. The typical pathological changes were characterized by extensive congestion and edema of hepatocytes, serious renal tubular epithelial cells degeneration and necrosis, focal lytic necrosis in the heart, renal interstitial edema and haemorrhage. In addition, degeneration, necrosis and abscission of the epithelium, congestion and oedema of the submucosa were noted in the intestinal tract. Main histopathological lesions in different organs are shown in Figure 2, respectively. No obvious pathological changes were found in the other organs.

### 4. Discussion

*Morganella morganii* is a facultative gram-negative and anaerobic rod and it may be a cause of devastating infections in neonates and immunocompromised hosts. *M. morganii* is a phenotypically conserved species, and little strain to strain variation in its biochemical characteristics are known to occur. The overall homogeneity of the phenotypic *Morganellae* might be recovered from specimens from sterile body sites using routine enteric isolation media (Janda *et al.*, 1996). For fecal specimens, Mac Conkey agar with methyl blue and phenolphthalein diphosphate might be helpful. *Morganella* strains might be maintained at room temperature in agar deeps, especially motility agar, for several months. Half of all *Morganellae* grown in broth culture showed uniform turbidity with ring or pellicle formation. Growth occurred between 4 °C and 45 °C. In this study, strain 602-1 was isolated from the sick Chinese giant salamander and its growing states, and morphological characteristics accorded with the features of *M. morganii*.

With the development of molecular genetics, alternative native molecular biological approaches have been developed in place of classic phenotypic methods for the identification of bacteria. Bacterial 16S rDNA amplifications had been widely used in the classification of uncultured bacteria inhabiting in environmental niches (Ajitkumar *et al.*, 2012; Vondracek *et al.*, 2011). With the introduction of high throughput sequencing methods in studies of microbial diversity, large datasets were rapidly accruing that allowed patterns of sequence recovery to be examined in depth across multiple habitats and samples (Jacob *et al.*, 2011). *M. morganii* was confirmed to cause a fatal infection in chickens by isolation and identification of the bacteria, 16S rDNA gene sequencing, and experimental infection (Zhao *et al.*, 2012). In this study, this isolate was verified to be *M. morganii*, based on the 16S rDNA analyses and morphological features.

There were some reports about the pathogenicity of *M. morganii* in humans and some animals (Kim *et al.*, 2007). The genome sequence of *M. morganii* provides important information concerning virulence and determinants of fitness. The pathogenicity-related genes identified in the *M. morganii* genome encode factors that influence virulence, such as fimbrial adhesins, flagellar structural proteins, haemolysins, ureases, and insecticidal and apoptotic toxins (Chen *et al.*, 2012).

A gastrointestinal stromal tumor abscess caused by *M. morganii* was diagnosed in an old man on the basis of radiological, microbiological, and histopathological findings (Chen and Lin, 2012). *M. morganii* had caused an infected abdominal aortic aneurysm in a 65-year-old man (Kwon *et al.*, 2011), and early-onset sepsis in a term neonate. Tubo-ovarian abscess caused by *M. morganii* was unusual and reported in a 54-year-old menopausal woman (Chou *et al.*, 2009).

*Morganella morganii* may be considered as a possible cause of septic arthritis in diabetic patients, especially those with diabetic foot infections (Cetin *et al.*, 2008). A case of leukemoid reaction complicating renal abscess caused by *M. morganii* infection was reported in an 80-year-old man. *M. morganii* was detected repeatedly in material of liquid from the
abscess and arterial blood culture (Osanai et al., 2008). In this study, the result indicated that the M. morganii strain 602-1 was pathogenic, which was in accord with the above reports. Of course, there were some differences of pathogenicity between various strains. In this study, severe pathologic changes mainly occurred in the liver, heart, spleen, intestine, and kidney of Chinese giant salamander, which verified that the strain 602-1 was highly virulent.

In conclusion, the identification and pathogenicity

Figure 1 Phylogenetic relationships of Morganella morganii strain 602-1, M. morganii, M. psychrotolerans, Citrobacter freundii, and C. murliniae.

Figure 2 Histology of Chinese giant salamander infected by Morganella morganii. A: The serious oedema of the hepatocytes and hyperaemia of hepatic sinusoids; B: The inflammatory cell infiltration between kidney tubules; C: The extensive hyperaemia and haemorrhagia of splenic sinus; D: The lytic necrosis in cardiac muscle fiber. Bars: A = 50 μm; B = 100 μm; C, D = 200 μm.
of *M. morganii* strain 602-1 were studied in the present study. This strain was pathogenic to Chinese giant salamander. Further investigations will be required to determine the pathogenic mechanism and prevention of *M. morganii* infections in *Andrias davidianus*.

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**References**


