Original Article

The characterization and pathogenicity of *Morganella morganii* strains isolated from beef cattle in Sichuan Province, China

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Abstract

Morganella morganii (*M. morganii*) is a member of the genera Morganella in the Enterobacteriaceae family. We tried to better understand the prevalence and characterization of *M. morganii* strains in cattle. A total of 191 nasal swab samples from beef cattle in Guang'an, Yibin, and Ziyang, Sichuan province, China were collected. The presence of *M. morganii* in the swabs was determined by PCR. Then positive swabs were processed using bacterial isolation and identification. The *M. morganii* isolates were assayed for antimicrobial susceptibility and pathogenicity using a mouse model. The prevalence of *M. morganii* in three cities was 10.5% (20/191). Three *M. morganii* strains (16GA7, 17GA61.2, and 17YB9) were isolated and identified from positive swab samples. Phylogenetic analysis demonstrated that the 16S rRNA gene of *M. morganii* isolates could be clustered with known Genbank *M. morganii* strains. All three *M. morganii* strains were sensitive to penicillin-type and quinolone antibiotics and were resistant to some cephalosporins, carbapenems, macrolide antibiotics and polymyxin B. Lesions in mice inoculated with *M. morganii* included pulmonary hemorrhage, thickened alveolar walls and pulmonary inflammatory cell infiltration, although lesions varied by strain. Through research on the *M. morganii* in beef cattle, it was found that mice were susceptible to *M. morganii* isolates. To the best of our knowledge, the present study is the first to report the prevalence of *M. morganii* in beef cattle.

Keywords: beef cattle, *M. morganii*, nasal swabs, pulmonary hemorrhage

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Introduction

M. morganii is a Gram-negative bacterium in the family *Enterobacteriaceae* that was first isolated in 1906 from a pediatric fecal sample (Morgan, 1906). This bacterium is part of the normal bacterial flora of humans and animals and is widely distributed in the environment (Bandy, 2020). *M. morganii* is an opportunistic pathogen that rarely causes infections but can be associated with a diverse number of conditions, including sepsis, abscessation, urinary tract infection, cellulitis, diarrhea and bacteremia (Katz *et al*., 2014; Salen and Eppes, 2014; Lin *et al*., 2015; Liu *et al*., 2016; Nakajima *et al*., 2017). *M. morganii* infections have been reported in a variety of domestic animals that are associated with humans, including broiler chickens, piglets, rabbits and dairy calves (Ono *et al*., 2001; Wani and Bhat, 2003; Zhao *et al*., 2010; McCarthy and Burkhardt, 2012; Lei *et al*., 2013; Li *et al*., 2013; Yu *et al*., 2013; Elfadl *et al*., 2017). It is noteworthy that *M. morganii* can live in the oral cavity of animals and thereby cause infections in humans via bites (Chunyi *et al*., 2009; Chen *et al*., 2011[a]; Fischer *et al*., 2016; Liu *et al*., 2016). Although the *M. morganii* can rarely cause the occurrence of zoonotic disease, it is noteworthy that *M. morganii* can spread in immunocompromised people (Di Ianni *et al*., 2015; Yacoub *et al*., 2015).

Since the 1970s, *M. morganii* has been considered an important cause of nosocomial infections in adults, such as urinary tract and wound infections (Lin *et al*., 2015). Some of these infections have had high mortality rates due to *M. morganii*'s virulence and increasing drug resistance (Lee and Liu, 2006). *M. morganii* has intrinsic resistance to glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, rifampicin, daptomycin, tetracyclines, colistin and nitrofurantoin (Zaric *et al*., 2021). *M. morganii* has been labeled an emerging "superbug" (Laupland *et al*., 2022). *M. morganii* infection can cause a range of different clinical symptoms in animals. *M. morganii* was isolated from chickens suspected with fowl typhoid or pullorum disease in Nigeria and important pathologic lesions, including enlarged and congested spleens, enlarged livers congested and friable areas with necrosis, congested lungs, congested, mis-shapened and atrophic ovaries (Mamman *et al*., 2006). Large numbers of *M. morganii* were isolated from the pneumonic lesion of a piglet with serofibrinous pleuropneumonia (Ono *et al*., 2001). *M. morganii* was isolated from the lungs, blood, liver, and blowhole mucosa of a dead dolphin with fibrino-hemorrhagic bronchopneumonia. This death was attributed to septicemia, based on the ecchymoses and petechiae of the spleen, pancreas, forestomach, lungs, visceral peritoneum and small intestine (Elfadl *et al*., 2017). Li *et al*. (2018), reported a case of *M. morganii* infection in Holstein calves in Tai'an, Shandong province that originated from lactating cattle with latent infections. In this instance, calves were infected from drinking milk and the presented clinical signs including depression, poor appetite, paralysis and feces with white flocs (Li *et al*., 2013). Little is known about the prevalence of *M. morganii* in Chinese cattle and this agent has not been previously reported in beef cattle.

Herein, prevalence of *M. morganii* in beef cattle was analyzed with three strains of *M. morganii* isolated from cow nasal swabs in Guang'an, Yibin and Ziyang, Sichuan province, China. Furthermore, the biochemical characteristics, drug sensitivity and pathogenicity of these *M. morganii* isolates were investigated.

Materials and Methods

All experimental procedures carried out in this study were approved by the Animal Ethical and Welfare Committee of Sichuan Agricultural University (No. SYXK20220189) following international standards, and care was taken to minimize the number of animals used.

Nasal swabs sample collection: From April 2016 to October 2019, nasal swab samples were collected from Sichuan province beef cattle farms located in Guang'an (n = 77), Yibin (n = 64) and Ziyang (n = 50) (Table 1). Samples were collected from cattle with recently developed clinical signs following transport from other provinces. Clinical signs included nasal discharge, cough and dyspnea.

Prevalence of M. morganii determined by PCR: DNA was extracted from the nasal swab sample using a One-tube General Sample DNAup for PCR kit (Sangon Biotech, Shanghai, China). The specific operation was carried out according to the instructions provided by the manufacturer and the extracted DNA samples were stored at -20℃ until analysis by PCR. The specific PCR primers Mm208F (5'-CTC GCA CCA TCA GAT GAA CCC ATA T-3') and Mm1017R (5'-CAA AGC ATC TCT GCT AAG TTC TCT GGA TG-3') were previously developed by Kim and others for detection of *M. morganii* based on the 16S rRNA sequence (Kim *et al*., 2006). Primers were synthesized by Sangon Biotech. 2×Master Mix (Qingke Zixi biotechnology, Chengdu, China) was used for amplification. *M. morganii* preserved in our lab was used as positive control in PCR. Pooled PCR products were confirmed by comparing DNA size with a DNA mass ladder (Qingke Zixi biotechnology Chengdu, China) on 1% agarose (Biowest, Spain) gel stained with ethidium bromide (Haoboyou biotechnology, Chengdu, China). Gels were placed on a Gel Documentation system (Bio-Rad, Hercules, CA, USA) for band visualization and photography.

Isolation and identification of M. morganii: Each *M. morganii* positive nasal swab sample was placed in Tryptic Soy Broth (TSB) medium (Hai Bo Biotechnology, Qingdao, China) for pre-enrichment. After incubation at 37℃ for 24 h, one loop of each pre-enriched culture was streak-inoculated on to blood agar and MacConkey plates (Hai Bo Biotechnology, Qingdao, China) and incubated at 37℃ for an additional 24 hr. According to Bergey 's Manual of Systematic Bacteriology (Krieg *et al*., 1984), suspected single colonies were selected for microscopic examination with gram staining. Next, the suspected *M. morganii* purification cultures were identified using bacterial trace biochemical identification tubes

(Microbial Reagent Co., Hangzhou, China) and PCR amplification. Primer synthesis and PCR products sequencing were conducted by Sangon Biotech using a 16S rRNA gene primer (F 5'-AGAGTTTGATCCTGG CTCAG-3', R 5'-TACGGCTACCTTGTTACGACTT-3'). Phylogenetic analysis was performed based on the 16S rRNA gene sequences available in the GenBank database. Phylogenetic trees were reconstructed using MEGA7.0 software (Sudhir *et al*., 2016), with sequences aligned to *M. morganii* reference strains retrieved from GenBank. Bootstrap values were tested with 1000 replicates using a neighbor-joining algorithm (NJ), and evolutionary distances were determined using the Kimura two-parameter model.

Antimicrobial susceptibility test: The Kirby-Bauer disk diffusion method was used to determine *M. morganii* isolate antimicrobial susceptibility. Three *M. morganii* isolates were tested to 16 antibiotics (ampicillin, carbenicillin, piperacillin, cefalexin, cefuroxime, imipenem, meropenem, streptomycin, kanamycin, amikacin, tetracycline, minocyline, midecamycin, enrofloxacin, florfenicol and polymyxin B, purchased from Microbial Reagent Co., Hangzhou, China) using the Kirby-Bauer disk diffusion method. Results were interpreted following Clinical and Laboratory Standards Institute document M100-S28 guidelines (Wayne, 2018).

Mouse challenge test: Pathogenicity of the isolated *M. morganii* strains was determined with Kunming mice (Da Shuo Laboratory Animal Co., Chengdu, China) challenge model. Mice were maintained in individual cages in a specific pathogen-free environment with an automatically-controlled 12-h light/dark cycle and free access to food and water for seven days. Mice were randomly assigned to four treatment groups with six mice in each group. Three treatment groups were inoculated with a unique *M. morganii* strain, and one group was set as control. Inoculations were performed using intraperitoneal injections of a suspension made from *M. morganii* cultures containing 4×10^8 CFU/mL (0.2 mL/per mouse) in a sterile common broth medium. Control group mice were intraperitoneally injected with equivalent volumes of sterile common broth medium. All mice were monitored for mortality and clinical signs such as ruffled fur, lethargy and dyspnea for 7 d post-inoculation. Mice that did not die after challenge with *M. morganii* were euthanized using sodium phenobarbital for sampling according to American Veterinary Medical Association guidelines (2013, AVMA) (Leary *et al*., 2013) and every effort was made to minimize animal suffering. Mouse hearts, livers, spleens, lungs and kidneys were collected aseptically post-mortem and fixed in 4% paraformaldehyde (Solarbio Life Science, Beijing, China) for routine processing and embedded in paraffin. Paraffin-embedded tissue samples were sectioned (3 μm) and stained with hematoxylin and eosin (H&E, Beyotime, Beijing, China). Organ histopathology of all mice was documented by two pathologists who were blinded to treatments.

Table 1 Formulation and chemical composition of the experimental groups (g/kg).

Sample types	Clinical syndromes	Farm location	Number of samples
Nasal swabs	Respiratory symptoms	Guang'an	77
Nasal swabs	Respiratory symptoms	Yibin	64
Nasal swabs	Respiratory symptoms	Ziyang	50

Note: respiratory symptoms including nasal discharge, cough, and dyspnea

Results

Prevalence of M. morganii: Analysis data of the 191 samples showed that the prevalence of *Morgenella morganii* in three cities was 10.5% (20/191), the prevalence in Guang'an city was 18.2% (14/77),with 7.8% (5/64) in Yibin and 2% (1/50) in Ziyang. The partial amplified positive PCR products were analyzed by agarose gel electrophoresis (Fig. 1). Single DNA positive bands were clearly shown on the gel.

M. morganii culture characteristics and 16S rRNA gene identification: Three *M. morganii* strains (16GA7, 17GA61.2, and 17YB9) were isolated from positive nasal swab samples. The strains of 16GA7 and 17GA61.2 were isolated from Guang'an city, 17YB9 was isolated from Yibin. Isolates grew as pale pink, translucent round small colonies on MacConkey medium (Fig. 2A) and as white, translucent, weakly beta hemolyzed round colonies on blood agar medium (Fig. 2B), belonged to gram-negative brevibacterium determined by gram staining (Fig. 2C). The 16S rRNA gene of the strains were amplified and sequenced. Through the NCBI BLAST analysis, the similarity between the obtained sequences of isolates and reference *M. morganii* sequences in Genbank could reach 99%. The 16S rRNA gene sequences of *M. morganii* strains (16GA7, 17GA61.2, and 17YB9) have been deposited in the GenBank database under accession numbers MN807692, MN807693, and MN807694. These strains were phylogenetically clustered with *M. morganii* strains SQ-1 (GenBank accession number KJ794191.1) and IT-4-1 (GenBank accession number KU570304.1) (Fig. 3). The three *M. morganii* strains had shared biochemical characteristics (Table 2); each strain could metabolize glucose, mannose, ornithine and phenylalanine, but could not metabolize sucrose, maltose, lactose, rhamnose, melibiose, arabinose, mannitol, sorbitol, adonitol, inositol, lysine, arginine, esculin, and salicin, and was also H_2S test negative. Semi-solid agar puncture test showed that three *M. morganii* strains had no motility.

Table 2 Biochemical test results for 16GA7, 17GA61.2, 17YB9 strains of *M. morganii*

Substrate	Results	Substrate	Results
Glucose	+	Mannitol	-
Sucrose	-	Sorbitol	-
Maltose	-	Adonitol	-
Lactose	-	Inositol	-
Mannose	+	Phenylalanine	+
Rhamnose	-	Ornithine	+
Melibiose	-	Lysine	-
Arabinose	-	Arginine	-
Semi-Solid Agar	-	Esculin	-
H_2S	-	Salicin	-

Table 3 Drug sensitivity test results for 16GA7, 17GA61.2, 17YB9 strains of *M. morganii*

Drugs	Inhibition diameters/mm			Sensitivity		
	16GA7	17GA61.2	17YB9	16GA7	17GA61.2	17YB9
Ampicilin	13.0	8.0	8.0	R	R	R
Carbenicilin	19.0	26.3	32.0	S-DD	S	S
Piperacilin	25.0	22.3	18.5	S	S	S-DD
Cephalexin	8.0	8.0	8.0	R	R	R
Cefuroxime	9.0	17.0	10.5	R	S-DD	R
Imipenem	14.0	13.5	10.0	R	R	R
Meropenem	15.0	16.0	14.0	S-DD	S-DD	R
Streptomycin	12.0	16.0	17.0	R	S-DD	S-DD
Kanamycin	11.0	19.8	22.0	R	S-DD	S
Amikacin	15.0	20.5	19.0	S-DD	S	S-DD
Tetracycline	23.0	11.0	21.8	S	R	S
Minocycline	18.0	9.5	20.7	S-DD	R	S
Midecamycin	8.0	8.0	8.0	R	R	R
Enrofloxacin	20.0	22.8	23.0	S	S	S
Florfenicol	8.0	25.3	24.3	R	S	S
PolymyxinB	8.0	8.0	8.0	R	R	R

Antimicrobial susceptibility test: Inhibition diameters and sensitivity for *M. morganii* strains 16GA7, 17GA61.2, and 17YB9 are shown in Table 3. Strain 16GA7 was sensitive to piperacillin, tetracycline, enrofloxacin and dose-dependently sensitive to carbenicillin, meropenem, amikacin and minocycline, and resistant to ampicillin, cephalexin, cefuroxime, imipenem, streptomycin, kanamycin, midecamycin, florfenicol and polymyxin B. Strain 17GA61.2 was sensitive to carbenicillin, piperacillin, amikacin, enrofloxacin, florfenicol and dose-dependently sensitive to cefuroxime, meropenem, streptomycin, and kanamycin, and resistant to ampicillin, cephalexin, imipenem, tetracycline, minocycline, midecamycin and polymyxin B. Strain 17YB9 was sensitive to carbenicillin, kanamycin, tetracycline, minocycline, enrofloxacin, florfenicol, dose-dependently sensitive to piperacillin, streptomycin, amikacin and resistant to ampicillin, cephalexin, cefuroxime, imipenem, meropenem, midecamycin and polymyxin B (Table 3).

Virulence of M. morganii in mice: Mice inoculated with *M. morganii* were depressed, huddled together, had abdominal breathing and sticky ocular secretions and moribund mice had open mouth breathing. No clinical abnormalities were evident in the control mice. All mice inoculated with strain 16GA7 dead, 3 mice inoculated with strain 17GA61.2 dead and no mice inoculated with strain 17YB9 dead. Pulmonary hemorrhage was evident in inoculated mice, with no other gross post-mortem pathology evident in other organs. Mice in the control group had no gross pathological abnormalities (Fig. 4). Each *M. morganii* strain was re-isolated from the tissue samples of mice inoculated with the corresponding strain.

Each *M. morganii* strain presented with similar histopathological findings, although differences were evident (Fig. 5). Strain 16GA7 was associated with pulmonary hemorrhage, alveolar wall thickening, pulmonary inflammatory cell infiltration, spleen congestion, hepatic cord disorder, hepatocyte swelling and necrosis and narrow renal cystic spaces. Strain 17GA61.2 was associated with pulmonary hemorrhage, necrosis, collapsed alveolar structures, pulmonary inflammatory cell infiltration, increased splenic multinuclear macrophages, indistinct splenic white pulp structure, hepatic cords derangement, hepatocyte swelling and necrosis and narrowing of the renal cystic cavity (Fig. 5). Strain 17YB9 was associated with pulmonary hemorrhage, alveolar wall thickening, pulmonary inflammatory cell infiltration, splenic congestion, vacuolar degeneration, hepatic cord derangement, hepatocyte swelling and necrosis, vacuolar degeneration, narrowing of the renal cystic space and vacuolar degeneration. There were no abnormalities in the cardiomyocytes of the mice infected with any of the *M. morganii* strains. There were no histopathological abnormalities in the control mice.

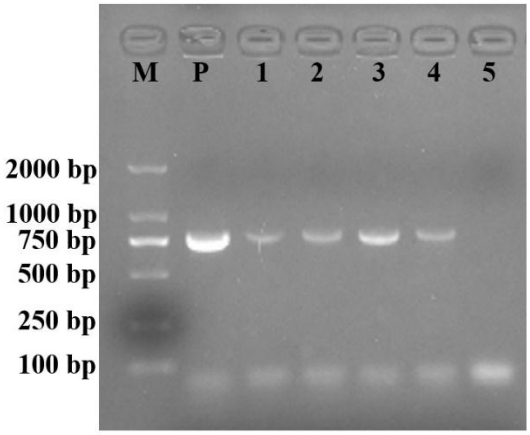

Figure 1 The partial amplified positive PCR products analyzed by agarose gel electrophoresis. M: DNA Marker, P: positive control, 1-4: samples PCR products, 5: negative control.

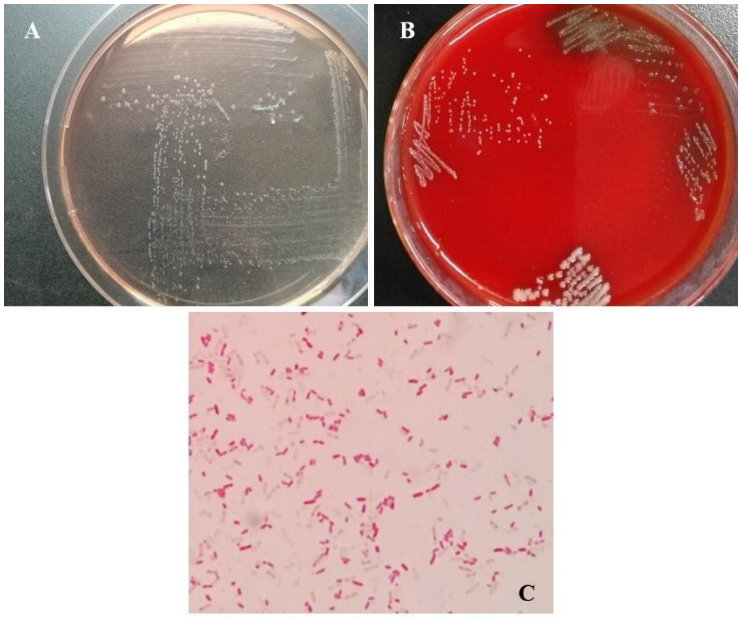

Figure 2 Morphology of *Morganella morganii* isolates. (A) Colony morphology of *M. morganii* strains in MacConkey agar. (B) Colony morphology of *M. morganii* strains in blood agar. (C) Microscopic morphology of *M. morganii* strains (1000 x).

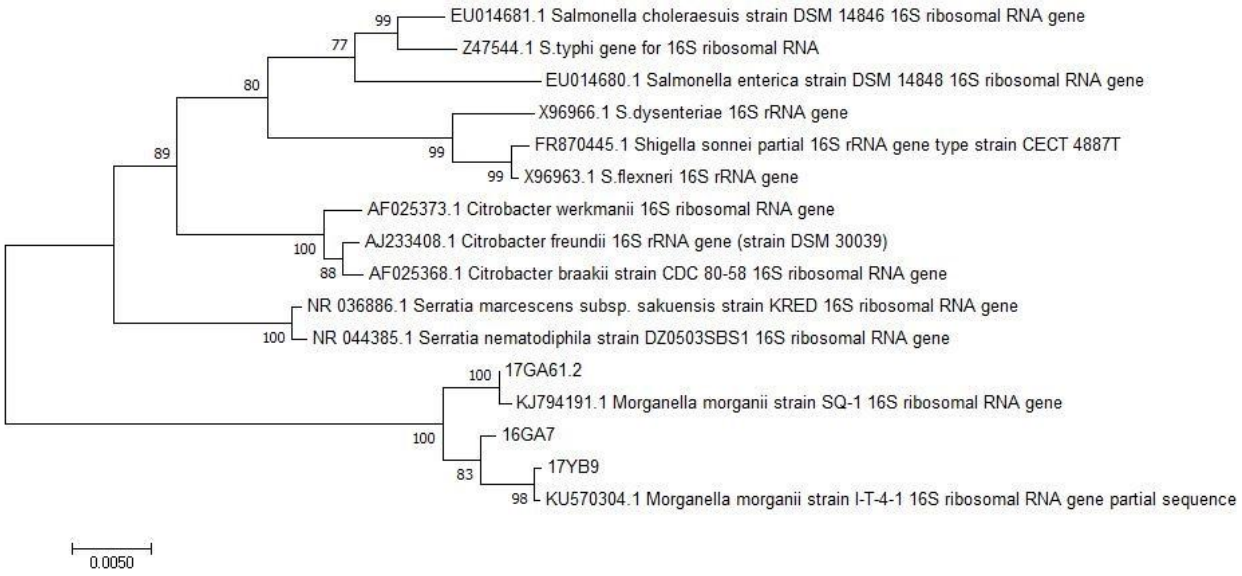

Figure 3 Phylogenetic tree analysis based on 16SrRNA gene sequences.

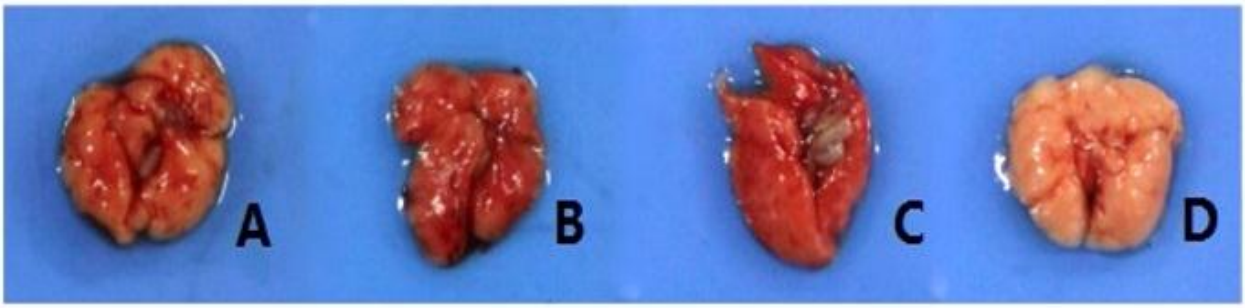

Figure 4 Representative gross post-mortem pulmonary tissues appearance for each treatment group. (A)16GA7, (B) 17GA61.2, (C)17YB9, (D) control group.

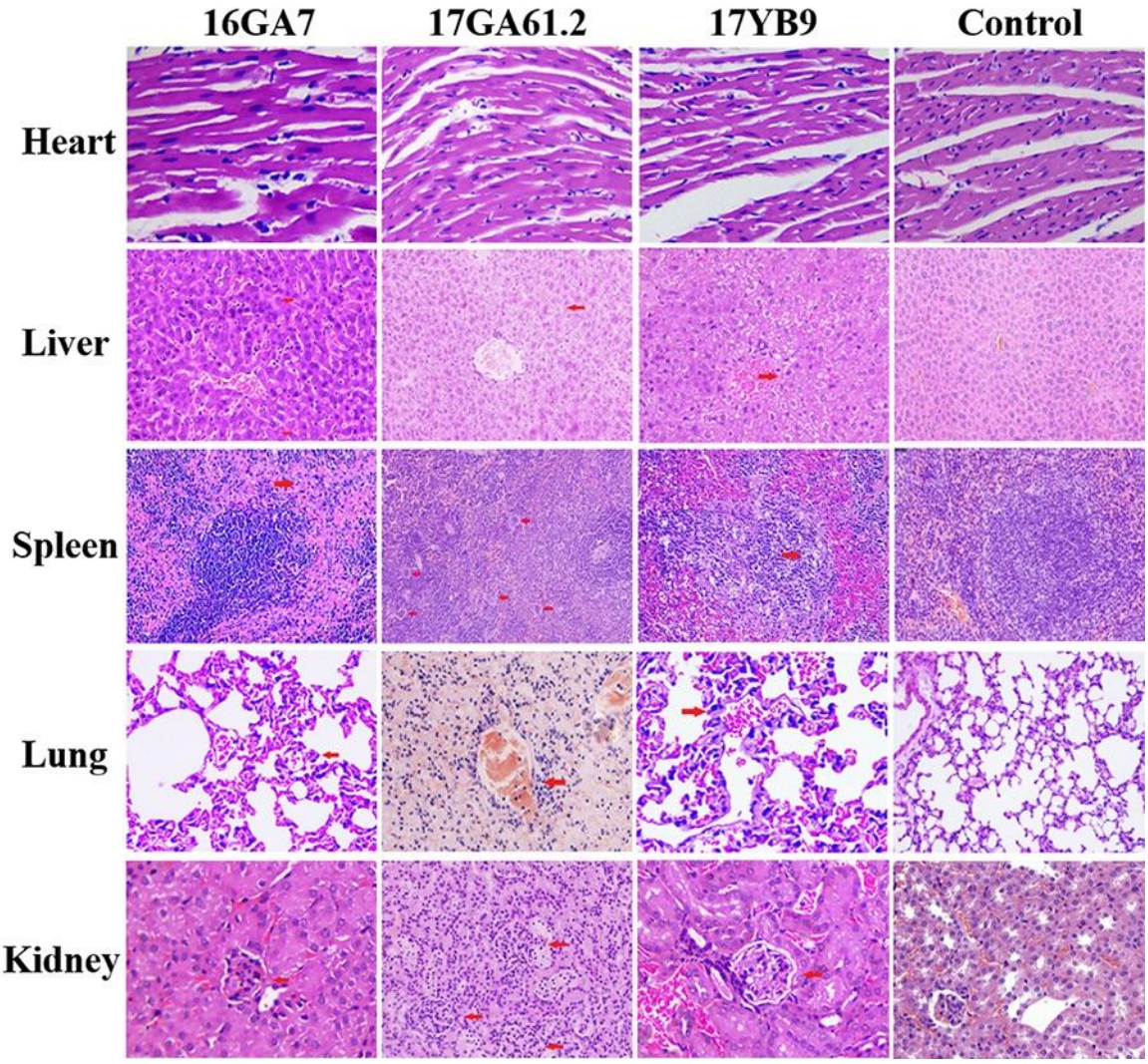

Figure 5 Representative photomicrographs of H&E-stained heart, liver, spleen, lung, and kidney sections (200x). Arrows for 16GA7, 17GA61.2, and17YB9 photomicrographs identify areas of hepatocyte necrosis. In addition to hepatocyte necrosis, livers from 17YB9 group have vacuolar degeneration. The arrow for the 16GA7 group's spleen indicates splenic congestion, whereas the arrows for the 17GA61.2 group's spleen indicates multinuclear macrophage proliferation, and the arrow for the 17YB9 group's spleen indicates splenic congestion and vacuolar degeneration. The arrows for the 16GA7 and 17YB9 groups in lung tissue indicate areas of pulmonary hemorrhage, alveolar wall thickening and pulmonary inflammatory cell infiltration, and the arrow for the 17GA61.2 group indicates pulmonary hemorrhage. The arrows for the 16GA7, 17GA61.2, and 17YB9 group's kidney photomicrographs indicate narrowing of the renal capsular space. In addition to narrowing of renal capsular space, the kidney for the 17YB9 group has vacuolar degeneration.

Discussion

M. morganii can be an opportunistic pathogen when the host's immune system is compromised and the agent spreads systemically (Earley *et al.*, 2016; Xiang *et al.*, 2021). *M. morganii* is widely distributed in nature and infections have been reported in fish, amphibians, reptiles, birds and mammals including humans (Liu *et al.*, 2016; Wang *et al.*, 2017). This agent has also been associated with food poisoning in humans (Papadopoulou *et al.*, 2007; Fernández-No *et al.*, 2010;

McCarthy and Burkhardt, 2012). Cattle farming has become a fast-growing industry in China. However, intensive beef cattle farming is facing challenges from infectious diseases. Our findings suggest that bovine *M. morganii* infections are a potential infectious disease concern for the cattle industry.

In this study, the overall prevalence of *M. morganii* in Guang'an, Yibin and Ziyang was 10.5% (20/191). This result suggests that the risk of exposure to *M. morganii* in cattle is high in Sichuan province. We isolated three *M. morganii* strains 16GA7, 17GA61.2 and 17YB9 from *M. morganii* positive nasal swabs collected from beef cattle farms in Sichuan province. According to the medical history investigation, these cattle had a history of long-distance transportation shortly prior to disease onset. Transportation-related stressors and may have compromised immunity in these animals, thereby favoring development of opportunistic *M. morganii* infections. A dolphin died from septicemia caused by *M. morganii* infection and transport stress and introduction to a new facility were also considered to be the most likely risk factors (Elfadl *et al*., 2017).

All three *M. morganii* isolates were sensitive to quinolone and some penicillin-type antibiotics and resistant to some cephalosporins, carbapenems, macrolide antibiotics and polymyxin B. The spectrum of antibiotics for which *M. morganii* has apparent resistance raises concerns for this agent becoming a multidrug-resistant or extensively drug-resistant zoonosis. The multidrug-resistant phenotype of these isolates makes it very concerning. A pediatric patient with sepsis was reported to have a *M. morganii* isolate that had NDM-1 and cephalosporinases, although this patient was managed successfully with a combination of aztreonam and ceftazidime-avibactam (Hobson *et al*., 2019). A Chinese cobra (*Naja naja atra*) isolate of *M. morganii* was reportedly susceptible to piperacillin (Wang *et al*., 2017). *Morganella morganii* isolated from cattle experiencing high mortality was sensitive to streptomycin, imipenem, aztreonam and cefoperazone (Li *et al*., 2013). Taiwanese patients had *M. morganii* isolates with resistance to first-generation cephalosporins and ampicillin-clavulanate (Lin *et al*., 2015). Based on our results, we recommended using enrofloxacin or amikacin for treatment. Differences between our results and previous *M. morganii* studies drug resistance studies (Li *et al*., 2013; Lin *et al*., 2015; Liu *et al*., 2016; Schultz *et al*., 2017; Hobson *et al*., 2019) may be caused by differences between individual strains and environment, including the application history of antibiotics.

The virulence of *M. morganii* strains 16GA7, 17GA61.2 and 17YB9 differed, as did the pathology associated with each strain. Among these three *M. morganii* strains, 16GA7 had the strongest virulence, followed by 17GA61.2 and with 17YB9 having the least virulence. Pathology documented in infected mice included pulmonary hemorrhage, thickened alveolar walls and pulmonary inflammatory cell infiltration, as well as varying degrees of splenic, hepatic and renal lesions. These results differ from previous results for sdta1-sdta5 *M. morganii* isolates that were associated with hepatic and renal necrosis, hepatic nodules and renal pelvis hemorrhage with the absence of pulmonary hemorrhage (Li *et al*., 2013). Comparative genomic analyses demonstrates that *M. morganii* pathogenesis varies with evolution of the agent's virulence (Chen *et al*., 2012[b]). Hosts are also likely to be more susceptible to *M. morganii* infection when they are immunocompromised. In short, differences among *M. morganii* isolates need to be further characterized for an improved understanding of pathogenicity, appropriate diagnostic screening, vaccine development and human public health control measures.

To the best of our knowledge, this study is the first to report the prevalence of *M. morganii* in cattle and only one case report about dairy calves previously addressed *M. morganii* risks (Li *et al*., 2013). Nevertheless, *M. morganii* infections may have existed in cattle for the long term without being diagnosed or misdiagnosed. Therefore, *M. morganii* infections are of concern for the potential to become an emerging disease threat to the Chinese commercial cattle industry. Thus, this study is important for understanding the prevalence and pathogenic characteristics of *M. morganii* and potential therapeutic antibiotics that may be effective for treating infections.

Nothing is perfect and neither is this study. There are some limitations. Firstly, the symptoms of disease were not recorded in detail for each cow in this study. Secondly, the dominant bacterium in the cattle samples of this study was *Morganella* and the weaker bacteria were not isolated and identified, which will result in an incomplete analysis of the causes of disease in sick cattle. These limitations will be improved in our future studies.

In this work, prevalence of *M. morganii* was surveyed and three *M. morganii* strains were isolated from beef cattle nasal swabs. In addition, our drug sensitivity assay results demonstrated that *M. morganii* was resistant to multiple antibiotics including cephalosporins, carbapenems, macrolide antibiotics and polymyxin B. Therefore, the development of appropriate management programs for *M. morganii* is vitally important and urgent for the Chinese commercial cattle industry.

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