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# Isolation and Characterization of *Vagococcus fluvialis* from Deceased Pigs in Jiangsu, China

**Zhixin Guan[1,2], Abdul Wahaab[2], Mohsin Nawaz[3], Muhammad Naveed Anwar[4], Yuhao Li[2], Junjie Zhang[2,5], Muhammad Muddassir Ali[6], Abdul Tawaab[7], Ali Ahmad Sheikh[8], Zhiyong Ma[2], Dengke Zhong[5]*, Mingfu Niu[1]* and Jianchao Wei[2]***

[1]*Henan University of Science and Technology, 263 Kaiyuan Road, Luoyang, 471023, China.*
[2]*Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China.*
[3]*Faculty of Veterinary and Animal Sciences, University of Poonch, Rawlakoot, Azad Jammu and Kashmir, Pakistan.*
[4]*Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan.*
[5]*Shanghai Vocational and Technical College of Agriculture and Forestry, Shanghai 201600, China.*
[6]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, 54000, Lahore, Pakistan.*
[7]*University of Agriculture, Faisalabad, Sub Campus Toba Tek Singh, 36050, Pakistan.*
[8]*Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan.*



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**Key words**
***Vagococcus fluvialis*, Isolation and identification, 16S rRNA sequencing, Minimum inhibitory concentration (MIC), Antimicrobials, Phylogenetic analysis**



## ABSTRACT

The Gram-positive *Vagococcus fluvialis* has been regarded as a potential bacterial pathogen. It has been isolated from the environment, birds, fishes, and mammals with a slight knowledge of their species. *V. fluvialis* is also believed to act as pro-biotic in fishes. However, in mammals, it is frequently isolated from infectious tissues, including on rare occasions from humans and livestock lesions. Pigs' infestation with *V. fluvialis* is rarely reported. Here, we have presented the first report of *V. fluvialis* isolated and characterized from deceased pigs in Jiangsu province of China. Three *V. fluvialis* strains were isolated from deceased pigs presenting different clinical symptoms between October 2020 to January 2021. The bacterial strains were identified by MALDI-TOF MS and were confirmed by 16S rRNA sequencing and biochemical profiling. Broth micro dilutions were used to assess the minimal inhibitory concentration of the antimicrobials of veterinary interest. The isolated strains all were resistant to clindamycin, erythromycin and tetracycline. The data described will be important to assess the epidemiology and tackle the likelihood of out-breaks associated with *V. fluvialis* across pig herds. It may also provide a basis to understand the behavior of various antimicrobial drugs against this potential pathogen, which could facilitate development of guidelines for combating the possible threats to the pig industry/herds in the region associated with this bacterium.

## INTRODUCTION

The *Vagococcus fluvialis* is a catalase-negative, Gram-positive bacterium, preliminary isolated in 1974 from river water and chicken feces. These isolates were formerly identified as a member of the genus *Lactococcus*; however, 16S rRNA gene sequencing modified their classification to a new genus i.e. *Vagococcus* (Collins *et al*., 1989). A second species, known as *Vagococcus salmoninarum*, was described following a molecular taxonomy investigation (Wallbanks *et al*., 1990). Even though they are separate, the genera *Vagococcus* and *Enterococcus* shared a tight

* Corresponding author: jianchaowei@shvri.ac.cn, mingfuniu@163.com, 211506@shafc.edu.cn
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evolutionary relationship, and some species are challenging to distinguish purely on phenotypic traits. There have only been a few reports of *V. salmoniarum* being isolated from sick salmonoid fish and rainbow trout (Schmidtke and Carson, 1994), and *V. fluvialis* being isolated in 1994 from the lesions of various mammals i.e., horses, pigs, cattle, and cats (Pot *et al*., 1994).

*V. fluvialis* was firstly reported to be associated with human infections in 1997 (from peritoneal fluid, wounds, and blood culture). However, these isolations were different from those which were isolated from the pig (unknown clinical source) both by SDS-PAGE profile and phenotypically (Teixeira *et al*., 1997). Until now, only a few studies have briefed the spreading of this bacterium in humans. One isolate from rare infective endocarditis and the other from a root-filled tooth which is associated with periarticular lesions (Al-Ahmad *et al*., 2008; Jadhav and Pai, 2019). *V. fluvialis* was also reported as a possible probiotic for fishes *in vitro* and *in vivo* and has been shown to defend against *Vibrio anguillarum* (a significant fish disease), and to have immunomodulatory effects on the host (Roman *et al*., 2012, 2013; Sorroza *et al*., 2012). It has a distinctive lipid pattern with a high concentration of D-alanylcardiolipin (Fischer and Arneth-Seifert, 1998). There are not many reports of *V. fluvialis* being the cause of sickness in pigs. Teixeira *et al* (Teixeira *et al*., 1997) identified and isolated several strains from multiple body organs, suggesting that this agent may contribute to opportunistic and systemic infection in pigs.

Fewer studies have described isolation, biological characteristics, and antibiotic resistance of isolated *V. fluvialis* strains, which may have great significance in epidemiology, clinical diagnosis, and control/therapy. Here, we reported the phenotypic and genotypic characteristics of three *V. fluvialis* isolates. We have constructed a 16S phylogenetic tree using other reported strains to establish their phylogenetic relationship with other available isolates. Furthermore, we have studied their susceptible/resistance against various available antimicrobials (through MIC) which all are still rare and vital information for this genus in the swine production system.

## MATERIALS AND METHODS

*Sample collection and bacteria isolation*

Forty-eight samples (containing heart, liver, spleen, lung, kidneys, brain, lymph node, and nasal swabs) were collected from six suddenly deceased fattening pigs (80-180 days old), with a history of dyspnea (information provided by a veterinary doctor of the farm). The sampled pigs were raised in three technified swine forms, located in two different counties of Jiangsu province in PR China. Simultaneously, 30 samples (ten from each farm, containing blood and nasal swabs (each five) were collected from the same farms as control from healthy pigs. After wiping the internal organs or bleeding points with a sterile inoculum ring, then each sample was directly streaked on Columbia agar (Difco and BBL, Detroit, MI, USA), with 5% sheep blood and incubated at 37°C for 24 h. One presumptive colony from each plate was inoculated in brain heart infusion medium (Difco and BBL, Detroit, MI, USA) supplemented with 5% fetal bovine serum and incubated at 37 °C for 24 h for further analysis. All the samples described above and prepared cultures were screened through PCR (Zeng *et al*., 2014; Sunaga *et al*., 2020) by using specially designed oligo dt primers (primer sequences are summarized in Table I) for 12 possible porcine respiratory pathogens (*Actinobacillus pleuropneumoniae* (App), *Bordetella bronchiseptica* (Bb), *Glaesserella parasuis* (Gps), *Pasteurella multocida* (Pm), *Streptococcus suis* (SS), *Mycoplasma hyopneumoniae* (Mh), porcine circovirus 2 (PCV2), pseudorabies virus (PRV), swine influenza A virus (IAVs), porcine reproductive and respiratory syndrome virus (PRRSV), porcine respiratory coronavirus (PRCV) and porcine hemagglutinating encephalomyelitis virus (PHEV).

*Species identification by MALDI-TOF-MS*

Species identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (VITEK MS, BioMérieux, Marcy-l' Etoile, France) according to the manufacturer's instructions. Bacterial mass spectra in the range of 2–20 kDa were acquired using a Microflex™ mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The results were checked through Myla software (MALDI Bio Typer™ 3.0). Two replicates of each sample were placed in plate wells and two readings were made for each sample. The obtained spectra were compared to the manufacturer's library and standard Bruker interpretative criteria were applied; scores of $\geq 2.0$ were accepted for species assignment and scores of 1.7-2.0 were accepted for genus identification.

*Identification of phenotypic characteristics by biochemical profiling*

The biochemical profiles of isolates were tested to confirm bacterial strain identification by the VITEK™ 2 automated identification system (BioMérieux, Hazelwood, MO, USA) according to the manufacturer's instruction. A VITEK™ 2 GP ID card was used for the identification of gram-positive bacteria.

**Table I. Primer pairs used to screen various bacterial and viral infections.**

| Virus/Bacteria | Target gene | Primer sequence (5'–3') |
|---|---|---|
| Porcine reproductive and respiratory virus (PRRSV) | *ORF7* | F: CCAGTTCCAGCCAGTCAATCA |
| | | R: GCCCCGATTGAATAGGTGAC |
| Porcine Circovirus 2 (PCV2) | *ORF2* | F: AAGGGCTGGGTTATGGTATG |
| | | R: CGCTGGAGAAGGAAAAATGG |
| Pseudorabies virus (PRV) | *gE* | F: ATGGGCATCGGCGACTACCT |
| | | R: CCACCGCCACAAAGAACACG |
| Porcine respiratory coronavirus (PRCV) | *Nucleocapsid* | F: AGCTATTGGACTTCAAAGGAAGATG |
| | | R: CATAGGCATTAATCTGCTGAAGGAA |
| Porcine hemagglutinating encephalomyelitis virus (PHEV) | *Spike protein* | F: CAACCAGATCCTTCCACATATAAAG |
| | | R: GAGCAATCATCCTCCACAAGA |
| Porcine cytomegalovirus | *gB* | F: CTCTCAAGAAGATGCCGTCTG |
| | | R: CTGCTGATATTCCAAGTGACGTA |
| Swine influenza A virus | *matrix (M) gene* | F: GGCTCTCATGGAATGGCTAAA |
| | | R: TGCAGTCCTCGCTCACT |
| *Actinobacillus pleuropneumoniae* | *omlA* | F: GGGGACGTAACTCGGTGATT |
| | | R: GCTCACCAACGTTTGCTCAT |
| *Glaesserella parasuis* | *CTinfF1* | F: CGACTTACTTGAAGCCATTCTTCTT |
| | | R: CCGCTTGCCATACCCTCTT |
| *Pasteurella multocida* | *Kmt1* | F: GGGCTTGTCGGTAGTCTTT |
| | | R: CGGCAAATAACAATAAGCTGAGTA |
| *Streptococcus suis* | *16S rRNA gene* | F: AGAAGAGTGGAAAGTTTCTCA |
| | | R: TCACAGTTTCCAAAGCGT |
| *Mycoplasma hyopneumoniae* | *p102* | F: GTCAAAGTCAAAGTCAGCAAAC |
| | | R: AGCTGTTCAAATGCTTGTCC |



*16S rRNA sequence determination and phylogenetic analysis*

Genomic DNA was extracted with the TIANamp stool DNA kit (TIANGEN, Beijing, China) in accordance with the manufacturer's protocol. The 16S rRNA sequences were amplified by PCR with primers (rDNA37f, 5'-AGAGTTTGATCCTGGCTCAGG-3', positions 8-37, and rDNA1479r, 5'-ACGGCAACCTTGTTACGAGTT-3', positions 1506-1479) (Twomey *et al*., 2012). The amplicons were purified using a PCR DNA Gel Purification kit (TIANGEN, Beijing, China) and sequenced by Sangon Biotechnology Co., Ltd. (Shanghai, China). The 16S rRNA sequences were edited with BioEdit Sequence Alignment Editor software version 7.2.6. The sequences obtained from this study were deposited in GenBank under accession numbers ON303629.1, ON307070.1, and ON307071.1. Phylogenetic analysis was performed using mega 7.0.26 through the maximum-likelihood method with reference sequences of *V. fluvialis* obtained from the GenBank database.

*Determination of the minimal inhibitory concentration*

The standard agar dilution method, as described by the Clinical Laboratory Standards Institute (CLSI, 2021; Humphries *et al*., 2021), was employed to determine the minimal inhibitory concentration (MIC) of *V. fluvialis* isolates against various 16 antimicrobial agents i.e., ampicillin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, clarithromycin, clindamycin, doxycycline, enrofloxacin, erythromycin, florfenicol, gentamycin, kanamycin, penicillin, tetracycline, and vancomycin (details summarized in Table III). All of these antimicrobials were purchased from the China Institute of Veterinary Drug Control (Beijing, China). As no breakpoint/threshold levels are known for *V. fluvialis*, antimicrobial resistance was assessed based on MIC values. *Staphylococcus aureus* strain ATCC25923 was used for quality control.

## RESULTS

*Pathogen identification*

On a global basis, co-infection of various bacterial and viral infections is very common in growing pigs and contributes to a wide range of polymicrobial diseases syndrome (van Dixhoorn *et al*., 2016; Wang *et al*., 2016; Kavanová *et al*., 2018; Saade *et al*., 2020; Zhao *et al*., 2020; Kang *et al*., 2022). The deceased pigs on thorough examination and post-mortem inspection showed signs of encephalitis, pneumonia, spleen enlargement, necrosis, haemorrhages, and consolidation in the lungs (Fig. 1).

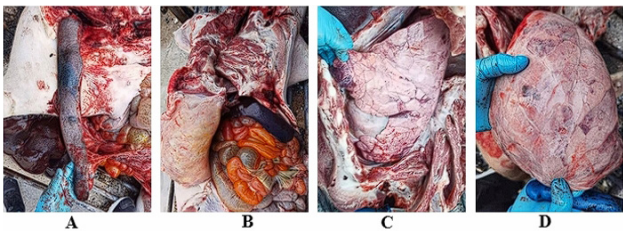


Fig. 1. Gross lesions of deceased pigs in Jiangsu province, China. The gross lesions observed in these dead pigs were mainly spleen enlargement, necrosis (A and B), lung hemorrhage and consolidation (C and D).


Fig. 2. Colony characteristics of suspected *Vagococcus fluvialis* in blood agar. All suspected *Vagococcus fluvialis* strains were formed small, pale, and smooth.

All the 78 samples (48 from dead pigs and 30 from healthy pigs) were screened for multiple bacterial and viral infections (details of diseases are summarized in Table I) through PCR (Zeng *et al*., 2014; Sunaga *et al*., 2020). Most of the samples were found negative. However, PCV2 was detected in various tissue samples from three deceased pigs and 40% of apparently healthy pigs from the same pen (data not shown). Three *V. fluvialis* strains (JS5, JS7, and JS12) were identified (by MALDI-TOF-MS) and isolated from the lungs and spleen of deceased animals with a confidence interval of more than 99% among them. All the strains were purified and formed small, pale, and smooth colonies in blood agar (Fig. 2).

*Phenotypic characteristics*

The biochemical profile of the three *V. fluvialis* strains (JS5, JS7, and JS12) was identified by VITEK™. The phenotypic characteristics of isolated strains are summarized in Table II. All strains produced acids from pyruvate, cyclodextrin, glycerol, mannitol, ribose, sorbitol, trehalose, and sucrose and failed to form acid from β-D-glucopyranoside, lactose, and raffinose. The strains were motile and grew in broth containing 6.5% NaCl at 10°C. The reactions for α-chymotrypsin, β-mannosidase, and pyroglutamic acid were positive among all studied strains, while reactions for N-acetyl-β-glucosaminidase, β-glucosidase and α-galactosidase were negative. The physiologic characteristics of all three *V. fluvialis* strains were similar to the description of *V. fluvialis* ATCC49515T (Fischer and Arneth-Seifert, 1998; Shewmaker *et al*., 2004). These findings suggest that all three isolated strains (JS5, JS7, and JS12) belong to the species *V. fluvialis*.

**Table II. Phenotypic characteristics of the isolated *V. fluvialis* strains.**

| Characteristic | ATCC 49515T# | JS5 | JS7 | JS12 |
|---|---|---|---|---|
| Growth in 6.5% NaCl | + | + | + | + |
| Growth in 10℃ | + | + | + | + |
| Growth in 45℃ | - | - | - | - |
| Motility | + | + | + | + |
| **Acid from:** | | | | |
| β-D-Glucopyranoside | - | - | - | - |
| Pyruvate | + | + | + | + |
| Cyclodextrin | + | + | + | + |
| Glycerol | + | + | + | + |
| Mannitol | + | + | + | + |
| Ribose | + | + | + | + |
| Sorbitol | + | + | + | + |
| Lactose | - | - | - | - |
| Trehalose | + | + | + | + |
| Sucrose | + | + | + | + |
| Raffinose | - | - | - | - |
| **Production of:** | | | | |
| N-Acetyl-β-glucosaminidase | - | - | - | - |
| α-Chymotrypsin | +W | +W | + | +W |
| β-Glucosidase | - | - | - | - |
| α-Galactosidase | v | - | - | - |
| β-Mannosidase | +W | +W | +W | +W |
| Pyroglutamic acidarylamidase | + | + | + | + |

- represents Negative, + represents positive, and +W represents weak positive.

*Sequence determination and phylogenetic analysis based on 16S rRNA*

The 16S rRNA sequences of the three isolated strains (JS5, JS7, and JS12) were deposited in the GenBank database through accession numbers ON303629.1, ON307070.1, and ON307071.1. A phylogenetic tree was constructed based on the 16S rRNA sequences using the neighbor-joining method (Fig. 3). Among the 16S rRNA sequences of 20 strains (including 17 reference strains gotten from the GenBank database), *V. fluvialis* JS5 has the highest similarity with *V. fluvialis* CCUG 32704 (Y18098.1) and *V. fluvialis* M19 (JF690756.1), both being 99.7% identical. *V. fluvialis* JS7 also has high similarity with them (both were 99.6% identical) and *V. fluvialis* JS12 also had the highest identity with *V. fluvialis* JS7, *V. fluvialis* GX20 (KU937383.1) and *V. fluvialis* GX21 (KU937384.1) with 99.3% similarity. *V.fluvialis* JS5 showed the highest divergence from *V. entomophilus* VOSTP2 (JQ 363620.1) (6.5%) and a slightly lower divergence from *V. salmoninarum* CCUG 3339 (Y18097.1) (6.4%). *V. fluvialis* JS7 showed the highest divergence from *V. salmoninarum* CCUG 3339 (6.5%) and a slightly lower divergence from *V. entomophilus* VOSTP2 and *V. humatus* C25 (KX247009.1) (6.2%). *V. fluvialis* JS12 had the highest divergence from *V. salmoninarum* CCUG 3339 (6.4%) and a slightly lower divergence from *V. entomophilus* VOSTP2 and *V. humatus* C25 (KX247009.1) (6.2%). Based on our 16S rRNA sequence analysis, all the isolated strains i.e JS5, JS7, and JS12 were identified as strains of *V. fluvialis* (Fig. 3).

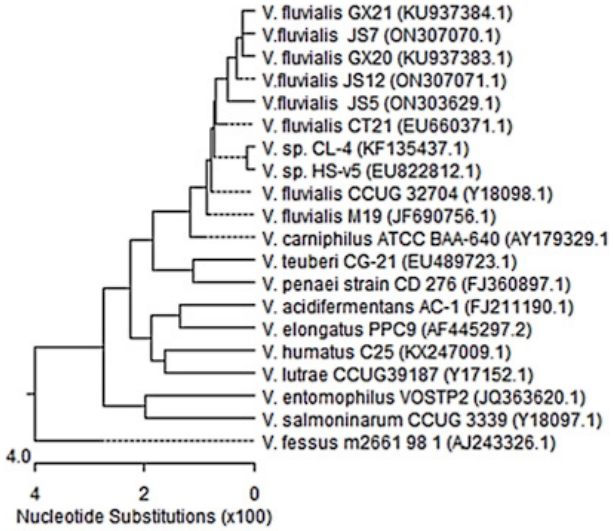


Fig. 3. Phylogenetic analysis of the isolated *V. fluvialis* strains.

*Antimicrobial susceptibility*

Sixteen antimicrobial agents were used to investigate their antimicrobial activities against *V. fluvialis* isolates (Table III). For the interpretation of the results of MIC,

**Table III. MIC (μg/mL) data and interpretive criteria of *V. fluvialis* strains from swine in China.**

| Antimicrobial agents | ATCC 25922 | JS5 | | JS7 | | JS12 | |
|---|---|---|---|---|---|---|---|
| | MIC (μg/mL) | MIC (μg/mL) | Interpretive criteria | MIC (μg/mL) | Interpretive criteria | MIC (μg/mL) | Interpretive criteria |
| Ampicillin | 5 | 8 | S | 0.5 | S | 0.5 | S |
| Ceftiofur | 0.25 | 2 | S | 1 | S | 1 | S |
| Ceftriaxone | - | 1 | I | 0.25 | S | 0.25 | S |
| Chloramphenicol | 5 | ≤1 | S | ≤1 | S | ≤1 | S |
| Ciprofloxacin | - | 4 | R | 4 | R | 2 | I |
| Clarithromycin | - | >2 | I | >2 | I | >2 | I |
| Clindamycin | - | >16 | R | >16 | R | >16 | R |
| Doxycycline | - | >16 | R | 4 | S | >16 | R |
| Enrofloxacyn | 0.025 | 0.25 | S | 0.5 | S | 0.25 | S |
| Erythromycin | - | 8 | R | 4 | R | 2 | R |
| Florfenicol | 5 | 2 | S | 8 | R | 4 | I |
| Gentamycin | 0.25 | 8 | R | 4 | I | 4 | I |
| Spectinomycin | 16 | 128 | R | 64 | S | 32 | S |
| Penicillin | - | 0.25 | S | 0.25 | S | 0.25 | S |
| Tetracycline | 1 | 64 | R | 32 | R | 64 | R |
| Vancomycin | - | ≤1 | S | ≤1 | S | ≤1 | S |

S, susceptible; I, intermediate; R, resistance.

**Table IV. *Vagococcus* species isolated from various sources.**

| *Vagococcus* species | Sample source | Health status | Sample type | Reference |
|---|---|---|---|---|
| *V. fluvialis* | Chicken | Healthy | Faeces and river water | Collins *et al*., 1989 |
| *V. salmoninarum* | Salmonid fish | Diseased | - | Wallbank *et al*., 1990 |
| *V. lutrae* | Lutra lutra | Healthy | Blood,liver,lungs and spleen | Lawsone *et al*., 1999 |
| *V. fessus* | Seal and harbour Porpoise | - | Liver and kidneys | Hoyles *et al*., 2000 |
| *V. carniphilus* | Cattle | - | Ground beef | Shewmaker *et al*., 2004 |
| *V. elongatus* | Pig | - | Swine-manure storage pit | Lawson *et al*., 2007 |
| *V. penaei* | Penaeus vannamei | - | Microbiota of cooked shrimp | Jaffrès *et al*., 2010 |
| *V. acidifermentans* | Acidogenic fermentation bioreactor | - | - | Wang *et al*., 2011 |
| *V. entomophilus* | Vespula vulgaris (*Wasp*) | - | Digestive Tract | Killer *et al*., 2014 |
| *V. martis* | Martes flavigula | - | Small intentine | Tak *et al*., 2017 |
| *V. humatus* | Pig | - | The soil beneath a decomposing pig carcass | Sundararaman *et al*., 2017 |
| *V. teuberi* | Malian sour milk fènè | - | Malian sour milk fènè | Wullschleger *et al*., 2018 |
| *V. bubulae* | Cattle | - | Ground beef | Shewmaker *et al*., 2019 |
| *V. vulneris* | Human | - | Foot wound | Shewmaker *et al.,* 2019 |
| *V. silagei* | Brewer's grain (*Silage production*) | - | - | Wu *et al*., 2020 |
| *V. xieshaowenii* | Snow finch (*Montifringilla taczanowskii*) | Healthy | Cloacal content | Ge *et al*., 2020 |
| *V. coleopterorum* | Diving beetle | Healthy | Intestine | Hyun *et al*.,2021 |
| *V. hydrophili* | Dark diving beetle | Healthy | Intestine | Hyun *et al*., 2021 |
| *V. zenguangii* | Yak (*Bos grunniens*) | Healthy | Faeces | Ge *et al*., 2021 |

- indicates no status given.



the cut-off points established by the Clinical and Laboratory Standards Institute (CLSI) were used for *Enterococcus* spp. except in the case of erythromycin, clarithromycin and ceftriaxone, in which those set for the group of Streptococci were used (Racero *et al*., 2021).

According to the CLSI, Among the 16 kinds of antimicrobial agents, three *V. fluvialis* were all sensitive to 6 kinds of antimicrobial agents, such as ampicillin, ceftiofur, chloramphenicol, enrofloxacin, penicillin and vancomycin. Among them, *V. fluvialis* JS5 was also sensitive to florfenicol, *V. fluvialis* JS7 was also sensitive to ceftriaxone, doxycycline and spectinomycin, *V. fluvialis* JS12 was also sensitive to ceftriaxone and spectinomycin. On the contrary, Three *V. fluvialis* were resistant to clindamycin, erythromycin and tetracycline. Among them, *V. fluvialis* JS5 was also resistant to ciprofloxacin, doxycycline, gentamycin and spectinomycin. *V. fluvialis* JS7 was resistant to ciprofloxacin. *V. fluvialis* JS12 was resistant to doxycycline. These data can provide effective suggestions for the use of drugs in pig farms infected with *V. fluvialis*. And shown that *V. fluvialis* strains are sensitive to most clinical antibiotics (see Table III for details) This indicated that *V. fluvialis* strains are susceptible to most clinical antibiotics (details are summarized in Table III).

## DISCUSSION

The genus *Vagococcus* was proposed in 1989 (Collins *et al*., 1989) to compensate for motile cocci which were identical to *Lactococcus* and were previously referred to as motile lactic streptococci. They were shown to be unique from all known *Lactococcus* (Schleifer *et al*., 1985). There are currently 20 known *Vagococcus* species that are isolated from a variety of sources including terrestrial, aquatic, insect species, and even animal products (details are summarized in Table IV) (Collins *et al*., 1989; Wallbanks *et al*., 1990; Lawson *et al*., 1999; Hoyles *et al*., 2000; Shewmaker *et al*., 2004; Lawson *et al*., 2007; Jaffrès *et al*., 2010; Wang *et al*., 2011; Killer *et al*., 2014; Sundararaman *et al*., 2017; Tak *et al*., 2017; Wullschleger *et al*., 2018; Shewmaker *et al*., 2019; Ge *et al*., 2020; Wu *et al*., 2020; Ge *et al*., 2021; Hyun *et al*., 2021). The 16S rRNA sequencing studies revealed that *Vagococcus* had a different and well-defined line of descent within lactic acid bacteria which represented a new species named *V. fluvialis* (Collins *et al*., 1989). Since its discovery, *V. fluvialis* have

been recovered from diverse sources including human clinical samples (peritoneal fluids, wounds, and blood) (Teixeira *et al*., 1997), river water and chicken feces (Hashimoto *et al*., 1974), and various domestic animals (lesions from cattle, cats, horses, pigs, and chickens (Pot *et al*., 1994) and could be regarded as a potential bacterial pathogen.

In this study, we have identified and characterized three distinct *V. fluvialis* strains (JS5, JS7, and JS12) that were isolated from deceased pigs in Jiangsu province of China, using their genotypic and morphological characteristics, biochemical profiles, and resistance to commonly used antimicrobial drugs.

Co-infection of various viral and bacterial infections is very common in growing pigs (van Dixhoorn *et al*., 2016; Wang *et al*., 2016; Kavanová *et al*., 2018; Saade *et al*., 2020; Zhao *et al*., 2020; Kang *et al*., 2022). The collected samples were further screened for twelve various diseases to instigate other possible reasons for death (detail of pathogens are summarized in Table I). Most of the samples screened were found negative for all of them. However, PCV2 was detected in various tissue samples from all deceased and fewer healthy pigs (40%) from the same pen suggesting that *V. fluvialis* may play a role in opportunistic infection (Teixeira *et al*., 1997; Nguyen *et al*., 2021). Though, its pathogenicity in pigs and synergistic mechanism with other pathogens need further studies.

Comparative 16S rRNA gene sequencing was performed to determine the phylogenetic affinities of the isolated strains with seventeen other strains gotten from Gene-Bank (Figure Phylogenetic tree) (Stackebrandt and Goebel 1994). Searches revealed that the gene sequences (16S rRNA) of *V. fluvialis* isolated strains JS5 and JS7 are phylogenetically most closely related to *V. fluvialis* M19 (JF690756.1), which was isolated from the midgut of Culexq unique fasciatus mosquitos in India (Chandel *et al*., 2013) and CCUG 32704 (Y18098.1) isolated by Lawson *et al*. (1999) (submitted). JS12 is closely associated with JS7 and strains GX20 (KU937383.1) and GX21 (KU937384.1), isolated from Guangxi, China in 2017. All the three isolated strains of *V. fluvialis* (J5, J7, and 12) showed a higher level of divergence (ranging from 6.2 to 6.5 %) with *V. entomophilus*, VOSTP2 (JQ 363620.1) isolated from the digestive tract of vasp (Killer *et al*., 2014), *V. salmoninarum* CCUG 3339 (Y18097.1) isolated by Lawson *et al*. (1999) (submitted) and *V. humatus* C25 (KX247009.1) isolated from the soil beneath a decomposing pig carcass in 2016 (Sundararaman *et al*., 2017).

Potential resistance to the chemotherapeutic drugs may be indicated by the proliferation of bacterial strains in the highest concentrations evaluated for the examined antimicrobials. Results of MIC determinations indicated that these *V. fluvialis* isolated strains were susceptible to ampicillin, ceftiofur, chloramphenicol, enrofloxacin, penicillin and vancomycin. However, they were found resistant to clindamycin, erythromycin, and tetracycline (details are summarized in Table III). Data obtained using the disc diffusion technique for *V. fluvialis* strains previously revealed that of the 18 microbial examined, the strain was found to be resistant to nafcillin, kanamycin, clindamycin, nalidixic acid, and norfloxacin (van Belkum *et al*., 2007). Teixeira *et al*. (1997) also provided MIC data for this species, which they reported as being resistant to lomefloxacin, ofloxacin, and clindamycin in a different investigation. The sensitivity of *V. fluvialis* isolated this study to clindamycin , erythromycin and gentamicin was different from the results of the article published by Garcia *et al*. (2016), The transition was from drug sensitivity to drug resistance, indicating that the sensitivity of *V. fluvialis* to several commonly used antibiotics is gradually decreasing, which will increase the risk of zoonosis.

This is the first report showing the spreading of *V. fluvialis* in deceased pigs of Jiangsu, China. The role of this bacteria in illness or death is so far unknown. However, it is significant to highlight that, none of the stains previously isolated from lesions in domestic animals (including cattle and pigs) encode for the genes associated with the virulence factor (Pot *et al*., 1994; Giannattasio-Ferraz *et al*., 2021), questioning their potential pathogenic association. Further *in vitro* and *in vivo* studies on the isolated strains would be very necessary to sort out whether isolated strains have any pathogenic and/or probiotic potential.



## CONCLUSION

Due to the possible zoonotic potential of the *Vagococcus* species, *V. fluvialis* may provide a risk, not only for swine production but also for pig industry workers. The findings in this study could be of great help in understanding the epidemiological behaviour of these neglected pathogens in the swine production system.

## DECLARATIONS

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*Ethics approval and consent to participate*

All animal experiments were approved by the Institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute, China (IACUC No: SVRI-P-2020101206) and performed in compliance with the Guidelines on the Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China, Policy No. 2006 398).

*Availability of data and materials*

All data generated during this study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/nuccore/ON303629.1, https://www.ncbi.nlm.nih.gov/nuccore/ON307070.1, https://www.ncbi.nlm.nih.gov/nuccore/ON307071.1. However, the raw data is available from the corresponding author upon reasonable request.

*Statement of conflict of interest*

The authors have declared no conflict of interest.

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