ORIGINAL ARTICLE

Characteristics of *Aerococcus viridans* isolated from porcine fetuses in Korean farms

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**Abstract**
Swine abortion caused by viruses as well as bacteria has caused many economic losses in domestic farms over the years; however, bacterial abortion has not yet been studied in Korea. Several bacterial species were isolated from aborted fetuses (*n* = 103) for which the cause of death was not viral abortion. Among them, we focused on *Aerococcus viridans*, which had the highest positive rate within three provinces (Gangwon, Jeonnam and Gyeongnam). A total of 16 isolates were identified as *A. viridans* by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and 13 were characterized by both antibiotic resistance and 16S rRNA gene analysis. Based on antibiotic susceptibility testing result, eight antimicrobials could not effectively eliminate the present isolation (more than 40% of isolates can resist these antibiotics), while all except two strains were susceptible to trimethoprim/sulfamethoxazole. Molecular analysis indicated genetic variation among these strains. This study is the first report detecting *A. viridans* from aborted fetuses in Korean domestic farms.

**KEYWORDS**
*Aerococcus viridans*, antibiotic resistance, phylogeny, porcine fetus
1 | INTRODUCTION

Abortion is a significant factor for economic losses in domestic farms. The causative agents of abortion in porcine fetuses can be divided into noninfectious and infectious causes. Viral infections such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), porcine circoviruses, encephalomyocarditis virus (EMCV), Japanese encephalitis virus (JEV), Aujeszky disease virus (ADV) and swine influenza virus (SIV) have normally been highlighted as the major infectious causes (Koenen & Vanderhallen, 1997; Zhang et al., 2015). However, infections with several bacterial species could be considered reasons for reproductive failure in swine. Bacteria associated with abortion in pig herds include Leptospira spp., Brucella suis and other opportunistic agents such as Escherichia coli, Streptococcus spp., Staphylococcus spp. and Erysipelothrix rhusopathiae (Givens & Marley, 2008; Vannier, 1999). A previous study indicated that pure bacterial factors accounted for approximately 24.5% of total infectious agents detected in fetuses from 2011 to 2013 (Salogni et al., 2016).

Aerococcus viridans is a Gram-positive cocci bacteria belonging to the Aerococcaceae family, Lactobacillales order. This species is considered an opportunistic bacteria that is relevant to several human infections, such as endocarditis, urinary tract infections, arthritis and meningitis (Ezechukwu et al., 2019; Nathavitharana et al., 1983; Taylor & Trueblood, 1985; Zhou et al., 2013). This species was also detected in clinical specimens from livestock (Martín et al., 2007; Pan et al., 2017; SAISHU et al., 2015) and wild animals (Colombo et al., 2021) with acute infections. In this study, several bacteria were isolated from samples collected over 2 years from aborted fetuses that did not have abortion-causing viruses detected. Among of them, we focused on A. viridans and characterized it through molecular biological analyses.

2 | MATERIALS AND METHODS

2.1 | Sample collection and bacterial isolation and identification

From January 2019 to May 2020, 103 fetal samples from different sows (2 fetuses per sow; 2019: n = 50 and 2020: n = 53) were separately collected from 25 farms (2019: n = 13 and 2020: n = 12) in six provinces (Gyeongnam, Gyeongbuk, Gangwon, Gyeonggi, Jeonnam and Chungnam) throughout South Korea (Figure S1). Pooled organs were homogenized and diluted 10-fold (w/v) with phosphate-buffered saline. These samples were confirmed as negative with eight reproductive-related viruses including: porcine circovirus type 2 (PCV2), PCV3, PRRSV, PPV, SIV, EMCV, JEV and ADV using the published primers (Nguyen et al., 2018) or commercial kits according to the methods of a previous study (Oh et al., 2017). Therefore, bacteria were isolated and purified on blood agar plus 5% defibrinated sheep’s blood. Pure colonies were maintained in stock containing glycerol at −70°C and then reactivated in brain–heart infusion (BHI) medium supplemented with fetal calf serum (5%) for 24 hr at 37°C for identification.

We conducted antimicrobial susceptibility testing of 13 isolated A. viridans fetus strains. The antimicrobial susceptibility to different commonly used antimicrobials was determined by the disk diffusion method using commercial disks (Oxoid, Ltd.). Nine antimicrobial disks were prepared: lincomycin/spectinomycin (109 μg), ceftiofur (30 μg), gentamicin (10 μg), oxytetracycline (30 μg), penicillin G (10 U), ampicillin (10 μg), trimethoprim/sulfamethoxazole (25 μg), amoxicillin/clavulanic acid (20/10 μg) and tiamulin (30 μg; Table 2). For the susceptibility test, 13 inoculated strains were prepared from a 48-hr Columbia blood agar plate by suspending four colonies in 5 ml of Mueller-Hinton broth and adjusting the solution to a 0.5 McFarland standard. The disk diffusion test was performed as described by the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards; Shryock, 2002) using Mueller-Hinton plates (Oxoid) supplemented with 5% defibrinated sheep’s blood (Martín et al., 2007). The inhibition zone diameter (IZD) breakpoint used was that recommended following the protocol for testing Staphylococcus (Martín et al., 2007; Standards, 2002), and the IZD breakpoint used was recommended by the CLSI (Lalitha, 2004; Watts, 1999).

2.3 | 16S rRNA sequencing of 13 isolated strains of A. viridans

DNA extraction was performed using a DNA/RNA extraction kit (iNtRON Biotechnology, Inc.). We amplified part of the 16S rRNA gene (Kozitskaya et al., 2005) using PfsF and PfsR primers. The PCR reaction mixture consisted of 2 μl of template DNA, 1 μl of each primer (10 μM) and 16 μl of Master mix solution (iNtRON Biotechnology, Inc.). The PCR thermal profile was initially denaturized for 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 2 min at 55°C, 1 min at 72°C and final extension for 7 min at 72°C. At approximately 1,400 bp, amplifying products were sent to a commercial facility (Macrogen Co., Ltd.) for sequencing.
2.4 | Phylogenetic tree construction and analysis

Including the 13 A. viridans strains from this study, the 16S rRNA genes from a total of 104 A. viridans strains were collected from GenBank to conduct a phylogenetic analysis. Using IQ-TREE v1.6.12 (Nguyen et al., 2015), the genetic relationships between A. viridans were inferred by the maximum likelihood (ML) method. The “-m TEST” option was used to help select data that automatically best fit the nucleotide substitution model. Branch support values were estimated by ultrafast bootstrap approximation (Hoang et al., 2018) implemented in IQ-TREE via the “-bb 1,000” option. The reconstructed phylogenies were displayed and the midpoint rooted by FigTree v1.4.3. To detect any potential recombination sites on the 16S rRNA gene of each A. viridans strain, Recombination Detection Program (RDP; version 4.460) was used (Martin & Rybicki, 2000) to compare the 13 A. viridans strains from this study with the 16S rRNA genes of 104 A. viridans strains collected from GenBank.

3 | RESULTS

3.1 | Bacterial isolation and phenotypic characteristics of A. viridans

In this study, 113 isolates of 46 different species were detected (Table 1). Of these, A. viridans was the most abundant species collected from fetuses (16 isolates) followed Enterococcus faecalis (14 isolates). Other commonly prevalent bacteria were Staphylococcus simulans (7 isolates), Camobacterium maltsomaticum (6 isolates), Lactobacillus sakei (5 isolates), Lactobacillus curvatus (4 isolates), Clostridium perfringens (4 isolates) and Streptococcus thoraltensis (4 isolates). Several other types of bacteria were also found (Table 1).

Focusing on A. viridans, this species was detected most commonly in Gangwon province (13 samples) followed by Jeonnam (three samples) and Gyeongnam (one sample). There were no A. viridans-positive samples in the remained three provinces. A. viridans growing on sheep blood agar showed circular yellowish colonies of a very small size of 1 mm, circular and several were α-haemolytic (Figure 1a). The microscopic characteristics of these isolates indicated that they were Gram-positive cocci measuring 1 μm in size that formed pairs or small groups (Figure 1b).

3.2 | Antibiotic susceptibility profiling of 13 isolated strains of A. viridans

Based on the IZD results, evaluation of these present isolates revealed that a high number of strains tolerated tiamulin (92.3%), amoxicillin/clavulanic acid (84.6%), lincomycin/spectinomycin (84.6%), ampicillin (76.9%), oxytetracycline (61.5%) and gentamicin (53.8%; Table 2). Nearly half of these isolates were resistant to ceftiofur (46.2%) and penicillin (46.2%; Table 2). On the other hand, trimethoprim/sulfamethoxazole tended to be effective for treating A. viridans, as only 15.4% of the strains were tolerant (Table 2).

### TABLE 1 Bacteria isolates from 103 fetuses from Korean domestic farms from 2019 to 2020

| Species identification               | 2019 (n = 50) | 2020 (n = 53) | Total | Prevalence |
|-------------------------------------|--------------|--------------|-------|------------|
| Aerococcus viridans                 | 7            | 9            | 16    | 15.5%      |
| Enterococcus faecalis               | 6            | 8            | 14    | 13.4%      |
| Staphylococcus simulans             | 1            | 6            | 7     | 6.8%       |
| Camobacterium maltsomaticum         | 2            | 4            | 6     | 5.8%       |
| Lactobacillus sakei                 | 5            | 0            | 5     | 4.9%       |
| Streptococcus thoraltensis          | 1            | 3            | 4     | 3.9%       |
| Lactobacillus curvatus              | 4            | 0            | 4     | 3.9%       |
| Clostridium perfringens             | 1            | 3            | 4     | 3.9%       |
| Escherichia coli                    | 0            | 3            | 3     | 2.9%       |
| Enterococcus avium                 | 0            | 3            | 3     | 2.9%       |
| Enterococcus pseudoavium            | 0            | 2            | 2     | 1.9%       |
| Staphylococcus aureus               | 0            | 2            | 2     | 1.9%       |
| Staphylococcus hyicus              | 0            | 2            | 2     | 1.9%       |
| Staphylococcus haemolyticus         | 1            | 1            | 2     | 1.9%       |
| Corynebacterium xerosis             | 2            | 0            | 2     | 1.9%       |
| Vegeococcus flavialis               | 2            | 0            | 2     | 1.9%       |
| Clostridium tertium                 | 0            | 2            | 2     | 1.9%       |
| Lactobacillus paracasei             | 0            | 2            | 2     | 1.9%       |
| Streptococcus dysgalactiae          | 0            | 2            | 2     | 1.9%       |
| Other bacteria                      | 11           | 18           | 29    | 28.2%      |
| Total                               | 43           | 70           | 113   |            |
Interestingly, whereas almost all of the isolated strains were resistant to at least five different types of antibiotics, isolates E9 and E13 were only tolerant to one or two types of antibiotics.

3.3 Phylogenetic tree analysis and potential recombinant origin of Korean A. viridans strains

A total of 13 strains of A. viridans were obtained accounting for approximately 1,400 bp of the partial 16S rRNA gene: W254 to W258, E3, and E8 to E14 (GenBank accession number. MT921600 to MT921611, MT928815). In the phylogenetic tree, the Korean strains showed genetic variation (Figure 2). Most of our isolates were close to the human-related strains or environmental origin strains, while W256 and E14 formed a separated branch in the phylogenetic tree. Eight strains (E3, E8, E10, E11, W254, W255, W267 and W25) were close to reference strains A.V04_China_2010_Human and A.V04_China_2015_Human. The E9_Korea_2020_Pig strain was genetically related to I17_China_2015_Air strain. In addition, the E12 and E13_Korea_2020_Pig strains were grouped with Clone_C040_Spain_2015_Sediment and were significantly different from the Korean isolates. The remaining two Korean strains (W256 and E14) each formed a different branch (Figure 2). We also detected several potential recombinant regions in the 16S RNA sequence of A. viridans isolates in this study (Table S1).

4 DISCUSSION

Viruses have been considered the main causes of swine abortion worldwide (Balasuriya & Carossino, 2017; Mak et al., 2018), but reports of bacterial abortion are scarce. This study was performed by collecting aborted fetuses in which the abortions were not caused by viral infection. Reproductive problems caused by bacteria are normally related to Brucella suis and Leptospira spp. (Vannier, 1999). However, in our study, A. viridans was highly prevalent, followed by Enterococcus faecalis and S. simulans (Table 1). A previous study classified these viruses into group I, which included facultative pathogens (Vannier, 1999). This study also suggested that, despite few reports focusing on opportunistic bacterial pathogens, the roles of this group in reproductive problems in pigs may be more common than is generally realized. For example, while Erysipelothrix spp. appeared to be quite common in healthy pigs, this genus still caused acute bacterial disease and induced reproductive diseases in sows (Opriessnig et al., 2020). Applying a vaccine strategy against this virus resulted in increased total born and live-born litter sizes in a Hungarian breeding unit (Hoffmann & Bilkei, 2002). Similarly, Staphylococcus spp. were isolated from the internal organs and fluids of aborted swine fetuses, suggesting its potential role in abortion in sows (Onet & Pommer, 1991; Salogni et al., 2016). Considering the absence of reproduction-related viral infection, isolated opportunistic pathogens were suggested to be the causative agents of swine reproductive disorders in the investigated farms.

In our study, the two most common bacteria detected in fetuses were A. viridans and Enterococcus faecalis. These species are commonly found in the urinary tracts of animals and may cause urinary/genital tract infection (Colombo et al., 2021; Ezechukwu et al., 2019; Strateva et al., 2016). However, the roles of these species in reproductive disorders are still under debate. Several species belonging to the Aerococcus genus were isolated in fetal organs after abortion (El-Arab et al., 2008; Twomey et al., 2008; Wolf-Jäckel et al., 2020). Focusing on A. viridans, this species has been reported to be associated with infection in humans (Ezechukwu et al., 2019; Nathavitharan et al., 1983; Taylor & Trueblood, 1985; Zhou et al., 2013) and animals (Devriese et al., 1999; Martin et al., 2007). A previous study indicated that this species was only isolated in the
herds with PRRSV (Martín et al., 2007). However, our study showed contrasting results that indicated that this species could be isolated in the clinical specimens without PRRSV infection. Nevertheless, our study showed high genetic diversity of *A. viridans* isolates (Figure 2), in agreement with the findings of previous studies (Martín et al., 2007; Nathavitharana et al., 1983). Environmental factors may have played a significant role in the divergence among hosts. The appearance in high frequency of this species in fetal organs might suggest the potential role of this species in swine reproductive diseases.

Our study has a number of limitations. First, this study did not analyse the relationship between symptoms of the sows and detection of abnormalities in fetal tissues. Additionally, environmental samples and cords from healthy piglets from the same farm were not collected. Thus, the possibility of *A. viridans* causing abortion in swine still needs to be verified. Abortions caused by environment bacteria are normally affected by environment influences and divergent among hosts. The lack of data about the presence of environmental bacteria are normally affected by environment influences and divergent among hosts. The lack of data about the presence of

| Antimicrobials | IZD* breakpoints | A. viridans-positive strains (in this study) | Resistant strains (%) |
|---------------|------------------|--------------------------------------------|----------------------|
| Lincomycin/Spectinomycin (109 μg) | ≤14 | W254 (+0), W255 (-16), W256 (+13), W257 (+11), E3 (+0), E8 (+0), E9 (+0), E10 (+0), E11 (+0), E12 (+0), E13 (+0), E14 (+0) | 84.6 |
| Ceftiofur (30 μg) | ≤17 | W254 (-29), W255 (-27), W256 (-29), W257 (+17), E3 (+0), E8 (+0), E9 (-25), E10 (+20), E11 (+16), E12 (-25), E13 (-27), E14 (-25) | 46.2 |
| Gentamicin (10 μg) | ≤12 | W254 (+0), W255 (+0), W256 (+0), W257 (-13), E3 (+0), E8 (+0), E9 (-18), E10 (-16), E11 (+0), E12 (-20), E13 (+0), E14 (-13) | 53.8 |
| Oxytetracycline (30 μg) | ≤15 | W254 (+13), W255 (+10), W256 (+12), W257 (-16), E3 (-26), E8 (-25), E9 (-18), E10 (+0), E11 (+12), E12 (+0), E13 (-18), E14 (+11) | 61.5 |
| Penicillin G (10 U) | ≤14 | W254 (-25), W255 (-26), W256 (-25), W257 (-20), E3 (+13), E8 (+0), E9 (-25), E10 (+0), E11 (+0), E12 (-25), E13 (+0), E14 (0) | 46.2 |
| Ampicillin (10 μg) | ≤16 | W254 (+15), W255 (+15), W256 (+14), W257 (-20), E3 (+14), E8 (+10), E9 (-24), E10 (+9), E11 (+0), E12 (-24), E13 (+0) | 76.9 |
| Trimethoprim/sulfamethoxazole (25 μg) | ≤12 | W254 (-27), W255 (-25), W256 (-25), W257 (+9), E3 (-13), E8 (-17), E9 (-15), E10 (-22), E11 (-16), E12 (-20), E13 (-15), E14 (-20) | 15.4 |
| Amoxicillin/Clavulanic acid (20/10 μg) | ≤13 | W254 (+0), W255 (+0), W256 (+0), W257 (+0), E3 (+0), E8 (+0), E9 (-23), E10 (+0), E11 (+0), E12 (-25), E13 (+0), E14 (0) | 84.6 |
| Tiamulin (30 μg) | ≤16 | W254 (+0), W255 (+0), W256 (+0), W257 (+0), E3 (+0), E8 (+0), E9 (-23), E10 (+0), E11 (+0), E12 (-25), E13 (+0), E14 (0) | 92.3 |
| Resistant/Total | 6/9 | 5/9 | 6/9 | 6/9 | 5/9 | 7/9 | 9/9 | 2/9 | 7/9 | 8/9 | 5/9 | 1/9 | 6/9 |

*Inhibition zone diameter (IZD) breakpoints.*
microbiological ecosystems in herds could limit the effects of these bacteria involved in abortion cases. Therefore, further case-control studies are needed to demonstrate the effect of this species in swine diseases.

In conclusion, *A. viridans* might be a potential pathogen related to abortion in pigs, but further studies focusing on its epidemiology and challenge experiments in sows must be pursued, and antimicrobial susceptibility testing of *A. viridans* in domestic pigs of all ages should be performed.

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**CONFLICT OF INTEREST**

The authors declare there is no conflict of interest.

**AUTHOR CONTRIBUTION**

Van Giap Nguyen: Data curation. Cheong Ung Kim: Conceptualization; Writing-original draft. Quynh Do Hai: Data curation; Writing-review & editing. Sook Shin: Data curation; Formal analysis. Keum Chan Jang: Formal analysis. Yong Ho Park: Conceptualization. Bong-Kyun Park: Conceptualization. Hee Chun Chung: Conceptualization; Supervision; Writing-original draft; Writing-review & editing.

**PEER REVIEW**

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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