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First isolation of *Aerococcus viridans* from clinical specimens collected on a pig farm in Poland

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Abstract

Introduction: Successful retrieval of *Aerococcus viridans* from porcine clinical specimens has been rarely described, and data has only been obtained from a few swine-producing countries. Therefore, the aim of this study was the isolation of *A. viridans* recovered from a specimen originating from a commercial pig farm located in Poland. **Material and Methods:** Seven dead 12-week-old pigs weighing 24–26 kg with joint swelling of the hind legs were selected on a modern farrow-to-nursery farm in Poland in October 2023. The research material was joint swabs from one affected limb amputated through the proximal part of the femur. Bacteria were isolated using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry. Antimicrobial susceptibility testing of isolates of *Staphylococcus aureus*, *A. viridans* and *Trueperella pyogenes* was performed by disc diffusion and also by minimal inhibitory concentration evaluation in the case of *A. viridans*. Two pooled samples were screened by PCR for *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Mycoplasma hyorhinis*, *M. hyosynoviae* and *Glaesserella parasuis.* **Results:** Five samples were positive for bacteria by culture and five isolates were recovered. *Staphylococcus aureus* was identified in three samples and *T. pyogenes* and *A. viridans* in one each. Pooled samples were *G. parasuis*-, *M. hyorhinis*-, *M. hyosynoviae*-, *Actinobacillus pleuropneumoniae*-, *Actinobacillus suis*- and *S. suis*-negative in the PCR. The *A. viridans* isolated was susceptible to beta-lactams and gentamicin. Ten representative nucleotide contigs from the 500 obtained showed similarity as high as 97.5% to GenBank reference strains. **Conclusion:** To the best of our knowledge, this is the first identification of *A. viridans* in clinically affected Polish pigs. It elevates the importance of uncommon pathogens, including *A. viridans*, in the development of lameness in pigs. This research emphasises the role of modern diagnostic tools for accurate identification of swine pathogens. Further research would prove beneficial to elucidate the mechanisms of *A. viridans* infection, its prevalence, pathogenicity and virulence factors.

Keywords: *Aerococcus viridans*, arthritis, pigs, Poland.

Introduction

Originally isolated from a hospital environment and described in the early 1950s, *Aerococcus viridans* was the first subspecies in the *Aerococcus* genus of the *Aerococcaceae* family and *Lactobacillales* order (39). Seven distinct Gram-positive cocci bacteria of the genus have been validly published to date: *A. christensenii*, *A. sanguinicola*, *A. suis*, *A. urinae*, *A. urinaeequi*, *A. urinaehominis* and *A. vaginalis* (1, 7, 12, 19, 20, 30, 39). Once regarded to be contaminants commonly present in air and soil (17, 18, 39), they are now deemed to be associated with different infections in humans. Even though the pathogenicity of the members of the genus has not been fully elucidated thus far, the bacteria have been infrequently reported as causative agents of arthritis (35), endocarditis (15, 28), meningitis (24) and urinary tract infections (2, 7, 8, 19).

Aerococcus spp. have also been reported to be pathogenic for different animal species. Initially, *A. viridans* was recognised as the causative agent of gaffkaemia, a fatal disease of lobsters (3, 13, 32).

According to some peer-reviewed studies analysing the problem of *A. viridans* infections in animals, the bacterium was subsequently associated with several cases of bovine mastitis (11, 21, 29, 31), septicaemia in immunodeficient laboratory mice (10), high mortality in a tilapia fishery (16), septicaemia in sea turtles (36) and genital infection in a captive elephant (9). Moreover, a growing body of evidence indicating the importance of *A. viridans* in a diverse spectrum of disorders in swine has been produced to date. The bacterium was successfully recovered from samples collected from pigs suffering from arthritis (22), meningitis (22, 27), pneumonia (22) and urinary tract infections (23), and most recently from aborted foetuses (25).

Peer-reviewed research works describing successful retrieval of *A. viridans* from clinical specimens collected from pigs are exceptionally rare and geographically limited to data only obtained from a few swine-producing countries. Similarly, reports delineating its antibiotic susceptibility and genetic diversity remain extremely scarce. Therefore, the aim of this study was to report the first isolation of *A. viridans* recovered from a specimen originating from a commercial pig farm located in Poland.

Material and Methods

Farm characteristics. The investigation was carried out in north-west Poland in October 2023 in a modern high-performing farrow-to-nursery farm with 5,000 DanBred sows and 22,000 weaners in place. All the pigs were reared on a slatted floor under conditions meeting the legal requirements for welfare of Council Directive 2008/120/EC of 18 December 2008 laying down minimum standards for the protection of pigs. The farm was using an all-in all-out system and reared pigs in weekly batches. The animals had uninterrupted access to barley- and wheat-based, dry-steam–conditioned (80°C) feed pressed into 4-mm-diameter cylindrical pellets. The levels of crude protein, fibre and fat in the feed offered to the sampled pigs were 17.21 %, 3.15 % and 3.95 %, respectively.

Health status of the animals. The herd was *Mycoplasma hyopneumoniae*-positive, porcine reproductive and respiratory syndrome virus (PRRSV)-negative, toxigenic *Pasteurella multocida*-negative, *Actinobacillus pleuropneumoniae*-negative, *Brachyspira hyodysenteriae*-negative, transmissible gastroenteritis virus (TGEV)-negative and porcine epidemic diarrhoea virus-negative. All the pigs received an intradermal vaccination against porcine circovirus 2-associated diseases and mycoplasmal pneumonia at the age of five weeks (Mhyosphere PCV ID; Hipra, Amer, Spain). The animals were vaccinated against intestinal lesions caused by *Lawsonia intracellularis* infection using Enterisol Ileitis (Boehringer Ingelheim Vetmedica, Ingelheim am Rhein, Germany) administered at the age of eight weeks.

Sample collection. For the purpose of the study, seven dead 12-week-old, 24–26 kg pigs with tarsal joint swelling were selected. The research material was obtained by a veterinarian by amputation of one of the affected limbs in the proximal part of the femur. The samples were randomly given numbers from 1 to 7 and conveyed overnight to SAN Group Biotech Germany (Höltinghausen, Germany) to be processed on the following day using the methods defined in the following descriptions.

Bacterial isolation and MALDI-TOF MS bacterial identification. Sterile swab samples for bacteriological culture were taken from the joint of each leg. They were plated onto six different agar plates (blood agar with and without *Staphylococcus* spp. nurse strain, neomycin blood agar, gentamycin blood agar, chocolate blood agar and bromothymol-blue lactose cystine agar) using a quadrant-streaking method. The plates were incubated at 37°C for up to 72 h. Every 24 h the plates were inspected for growth. Bacterial isolates were identified by matrix-assisted laser desorption/ ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) performed on a MALDI Biotyper (Bruker Daltonik, Bremen, Germany) according to the manufacturer's instructions. Parallel to this, a *Mycoplasma* enrichment was carried out using special enrichment medium for swine (Mycoplasma Liquid Medium; Mycoplasma Experience, Bletchingley, UK). Enrichment cultures were incubated for four weeks at 37°C and checked weekly for growth of *Mycoplasma* spp.

Antimicrobial susceptibility testing. Disc diffusion testing established the antimicrobial susceptibility of *Staphylococcus aureus*, *A. viridans* and *Trueperella pyogenes*. The special interest taken in *A. viridans* also prompted the determination of the minimal inhibitory concentration (MIC) of antimicrobials against this bacterium. Antimicrobial susceptibility testing was carried out according to the methods described in the Clinical and Laboratory Standards Institute VET01S protocols using adequate agars for the respective bacteria (6).

Polymerase chain reaction (PCR). Joint swabs were pooled for PCR testing, one pool comprising the swabs from four legs and the other the swabs of three. Extraction of DNA in the swabs was performed by routine molecular biological methods using a Kylt RNA/DNA Purification Kit (SAN Group Biotech Germany, Höltinghausen, Germany). Real-time screening PCRs for *Streptococcus suis* (Kylt Streptococcus suis, SAN Group Biotech), *Actinobacillus pleuropneumoniae* (Kylt Actinobacillus pleuropneumoniae, SAN Group Biotech), *Actinobacillus suis* (EXOone Actinobacillus suis oneMIX qPCR kit; EXOPOL, Zaragoza, Spain), *M. hyorhinis* and *M. hyosynoviae* (Kylt MHRS TRIPLEX, SAN Group Biotech), and *Glaesserella parasuis* (Kylt GPS, SAN Group Biotech) were performed according to the manufacturers' instructions.

Biomolecular identification. Whole-genomic sequence analysis was conducted by SAN Group Biotech Germany using in MEGA11 (34) and Geneious Prime software (Biomatters, Auckland, New Zealand). Nucleotide alignment aided by the Geneious alignment algorithm was performed after a National Center for Biotechnology Information BLAST search of 10 the most representative contigs. The obtained contigs were aligned to the two most similar sequences of *A. viridans* from the GenBank database, and the final sequences were submitted to the database (publication in progress).

Results

Of the seven samples examined, five were positive for bacteria by culture and five bacterial isolates were successfully recovered. *Staphylococcus aureus* was identified in samples 1, 2 and 6. *Trueperella pyogenes* and *A. viridans* (Fig. 1) were isolated from samples 2 and 7, respectively. Samples 3 and 5 were negative for bacterial growth. Sample 4 showed an overgrowth by *Proteus* sp. The two pooled samples were *G. parasuis*-, *M. hyorhinis*-, *M. hyosynoviae*-, *Actinobacillus pleuropneumoniae*-, *Actinobacillus suis*- and *S. suis*-negative in the real-time PCR.

The results of the antibiotic susceptibility testing for *A. viridans* are presented in Table 1 as minimal inhibitory concentrations. The isolate obtained in this study was susceptible to beta-lactam antibiotics (amoxicillin-clavulanic acid, ampicillin, ceftiofur and penicillin) and gentamicin. The isolate presented intermediate susceptibility to enrofloxacin and florfenicol, and was resistant to all the other tested antimicrobials.

The conducted analysis of 10 representative nucleotide contigs from the 500 obtained (which ranged in length from 700 base pairs (bp) to 22 kbp) showed similarity ranging from 96.6 % to 97.5% to the reference strains obtained from the GenBank database (Fig. 2).

The conducted phylogenetic analysis showed the occurrence of four main groups of *A. viridans* genomic sequences (Fig. 3). The similarity of the obtained genomic sequences of this bacterium ranged from 96.1% to 100%, indicating the high similarity of all the compared isolates. The first group was formed by the sequences contig_499, contig_19 and contig_443. The second group consisted of the contig_487 and contig_447 sequences, of which the sequence similarity reached 100%. The third group held the contig_483, contig_447 and contig_479 sequences, where the sequence similarity was 97.1–99.0%. The fourth group was comprised of five reference strains obtained from the GenBank database and the contig_12 and contig_462 sequences. The latter *A. viridans* sequence showed 100% homology to sequences from the GenBank database.

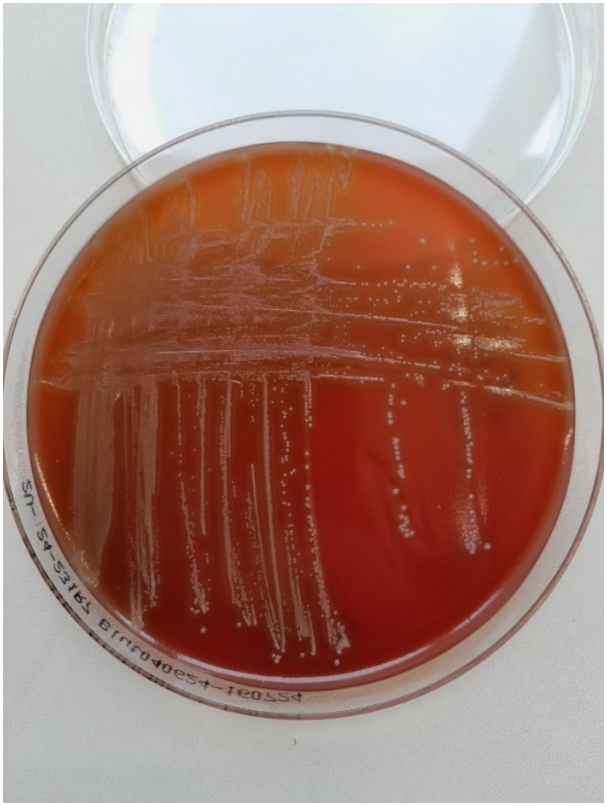

Fig. 1. Blood agar–cultured *Aerococcus viridans* isolated from a joint swab taken from the hind leg of a 12-week-old pig with tarsal joint swelling

Table 1. Determination of minimal inhibitory concentrations (μg/mL) of antimicrobials against *Aerococcus viridans* isolated from a joint swab taken from the hind leg of a 12-week-old pig with tarsal joint swelling

Antibiotic	MIC (μg/mL)
Amoxicillin/clavulanic acid	S (≤1/0.5)
Ampicillin	S (≤0.03125)
Ceftiofur	S (≤0.125)
Colistin	R (>2)
Enrofloxacin	I (=0.5)
Erythromycin	R (>4)
Florfenicol	I (=4)
Gamithromycin	R (>8)
Gentamicin	S (=1)
Penicillin	S (=0.125)
Trimetoprim/sulfamethoxazole	R (>2/38)
Tetracycline	R (>8)
Tildipirosin	R (>32)
Tiamulin	R (>16)
Tilmicosin	R (>16)
Tulathromycin	R (>64)

MIC – minimal inhibitory concentration; S – susceptible; I – intermediate susceptible; R – resistant

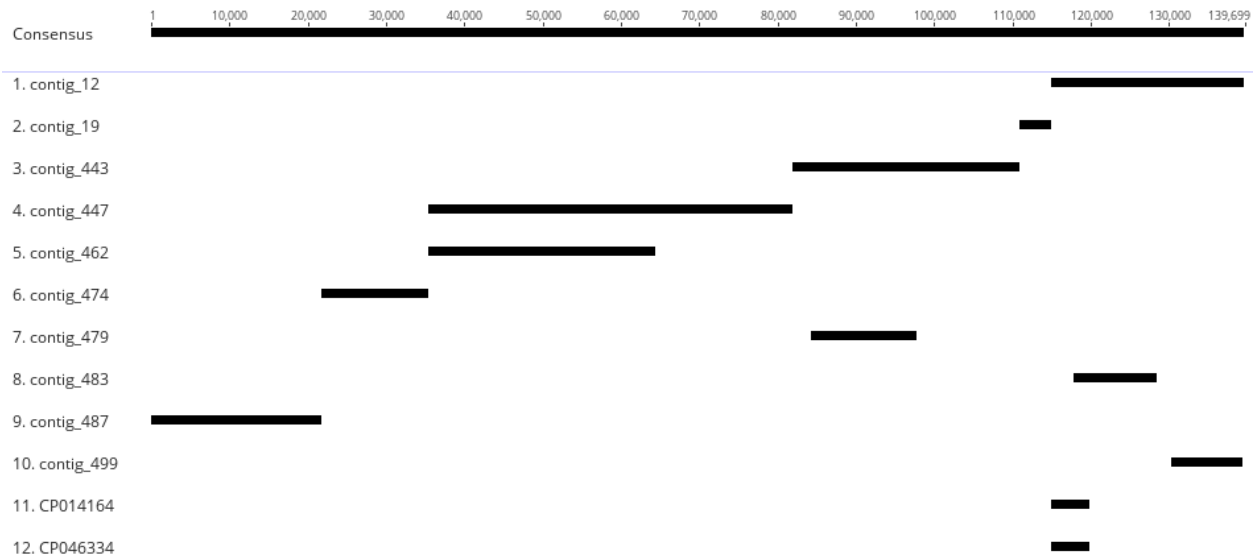

Fig. 2. *Aerococcus viridans* reference sequence nucleotide alignment of 10 representative contigs isolated from a joint swab taken from the hind leg of a 12-week-old pig with tarsal joint swelling. CPnnnnnn – GenBank reference strains of *A. viridans*

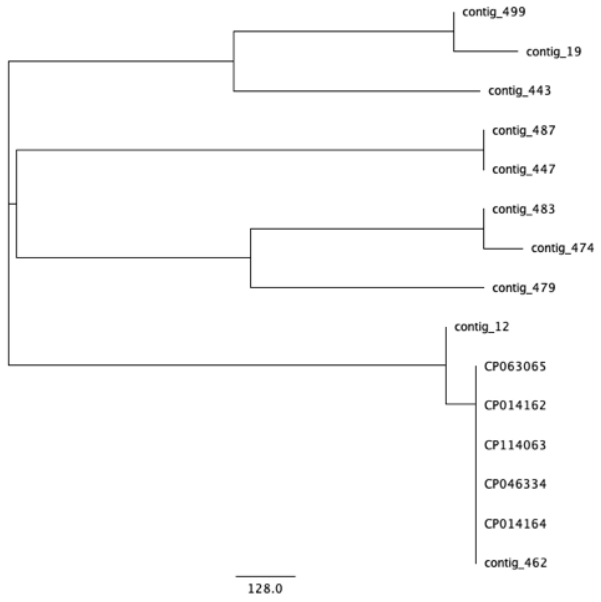

Fig. 3. Phylogenetic tree showing distances between reference sequences of *Aerococcus viridans* from the GenBank database and the obtained contig nucleotide sequences isolated from a joint swab taken from the hind leg of a 12-week-old pig with tarsal joint swelling. CPnnnnnn – GenBank reference strains of *A. viridans*

Discussion

The phenotypical similarity of *A. viridans* to streptococci and staphylococci could have led to the misidentification and considerable underestimation of the prevalence of infection with this bacterium in clinical veterinary practice worldwide. Our findings corroborate data indicating that *A. viridans* should be considered a pathogen which can potentially produce arthritis in pigs. Though the complex mechanisms leading to disease development have not yet been satisfactorily elucidated, the very first isolation of the bacterium from swine herds most of which had been exposed to PRRSV infection seems to support the role of immunosuppressive factors in the pathogenicity of *A. viridans* (22). The contribution of immunosuppression to the development of clinical symptoms of sepsis has been scientifically proven using a murine model (10). Consequently, some less obvious determinants could have facilitated the development of the *A. viridans*-associated arthritis in the pigs sampled for our research; nonetheless, the samples were obtained from a high-health-status, PRRSV-negative farm, and identification of other factors which may have triggered or exacerbated the symptoms was beyond the scope of our study.

Most of the members of the *Aerococcaceae* family are sensitive to beta-lactam antibiotics. Regardless of the documented existence of beta-lactam resistant clinical *A. viridans* strains in humans (4, 5, 33, 37) and in cattle (31), the isolate obtained in our investigation was susceptible to the antimicrobial class in question. Unfortunately, few data regarding the antibiotic susceptibility of *A. viridans* recovered from clinically affected pigs have been published to date. Moreover, different techniques and sets of antimicrobials were applied to evaluate their biological properties. The available research works describe the susceptibility patterns of Spanish, Brazilian and Korean isolates obtained from various swine clinical specimens (arthritis, pneumonia and meningitis) (22), urinary tract infection (23) and foetuses (25), respectively. In the first two studies, *A. viridans* clinical strains were found to be either susceptible to beta-lactams (*i.e.* amoxicillin-clavulanic acid, ampicillin, ceftiofur and penicillin) using the disc diffusion method (22) or to be inhibited by low MIC values of the same class of antibiotics (23) when the technique was not disc diffusion. The third piece of cited research reported a relatively high incidence of beta-lactam resistance amongst *A. viridans* isolates using the disc diffusion method, with amoxicillin-clavulanic acid-, ampicillin-, ceftiofur- and

penicillin-resistant strains comprising 84.6 %, 76.9 %, 46.2 % and 46.2 % of the total isolates, respectively (25).

Gentamicin had a low MIC against the isolate obtained in our study (1 µg/mL). The result was comparable with the MIC_{50} of ≤1 µg/mL and MIC_{90} of 2 µg/mL reported in the Brazilian study for this antibiotic against *A. viridans* strains (23). The intermediate MIC value obtained for florfenicol of 4 µg/mL was significantly lower than the MIC_{50} of 8 µg/mL described in the same work. Contrary to the previously reported antimicrobial susceptibility data, enrofloxacin was found to have a lower value against the isolate of 0.5 µg/mL, determining it to have intermediate efficacy. The elevated MIC values for macrolides and tetracycline determined in our work are comparable with those obtained in other studies addressing the problem in livestock (21, 26).

The major issue limiting our investigation may be the samples having been collected from a relatively low number of animals originating from one location only; nevertheless, the main study objective was to report the first isolation of *A. viridans* from a clinical specimen obtained from a pig farm located in Poland, and, since no other research questions were addressed, the sample size should be considered irrelevant. The isolation described herein might have been directly related to a microbiological contamination, because the bacterium has been described as saprophytic and successfully recovered from environmental samples (14, 38, 39). Nevertheless, since none of the major contagious agents generally associated with the development of infectious arthritis in swine (*i.e. M. hyosynoviae*, *M. hyorhinis*, *G. parasuis*, *S. suis*, *Erysipelothrix rhusiopathiae*, *Actinobacillus suis*, *Actinobacillus pleuropneumoniae* and *T. pyogenes*) were detected in the *A. viridans*-positive samples, and compelling evidence on the role of *A. viridans* infections in livestock has already been provided by other authors, its recovery from a normally sterile body part of a clinically affected animal can be considered indicative of the clinical relevance of the case being discussed.

Conclusion

To the best of our knowledge, this is the first official report of the identification of *A. viridans* in a clinically affected pig reared in Poland. The results of our investigation should raise awareness of the importance of uncommon pathogens, including *A. viridans*, in the development of lameness in pigs. Moreover, the presented research emphasises the vital role of modern diagnostic tools allowing the accurate and rapid identification of swine pathogens. Since no concrete details of the mechanisms of *A. viridans* infection, its prevalence, pathogenicity or virulence factors have yet been given, further research would prove beneficial.

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Animal Rights Statement: Ethical review and approval were waived for this study, as the analysed material originated from a routine diagnostic procedure ordered by the farm owners.

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