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Original Article

Vagococcus sp. a porcine pathogen: molecular and phenotypic characterization of strains isolated from diseased pigs in Brazil

Carlos Emilio Cabrera Matajira[1], André Pegoraro Poor[1], Luisa Zanolli Moreno[1,2], Matheus Saliba Monteiro[1], Andressa Carine Dalmutt[1], Vasco Túlio Moura Gomes[1], Mauricio Cabral Dutra[1], Mikaela Renata Funada Barbosa[3], Maria Inês Zanolli Sato[3], Andrea Micke Moreno[1]

[1] Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil
[2] Max Planck University Center (UniMax), Indaiatuba, Brazil
[3] Environmental Company of the State of São Paulo (CETESB), São Paulo, Brazil

Abstract

Introduction: *Vagococcus* spp. is known for its importance as a systemic and zoonotic bacterial pathogen even though it is not often reported in pigs. This is related to the pathogen misidentification due to the lack of usage of more discriminatory diagnostic techniques. Here we present the first report of *Vagococcus lutrae* in swine and the characterization of *Vagococcus fluvialis* and *Vagococcus lutrae* isolated from diseased animals.

Methodology: Between 2012 and 2017, 11 strains with morphological characteristics similar to *Streptococcus* spp. were isolated from pigs presenting different clinical signs. Bacterial identification was performed by matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry and confirmed by 16S rRNA sequencing and biochemical profile. Strains were further genotyped by single-enzyme amplified fragment length polymorphism (SE-AFLP). Broth microdilution was used to determine the minimal inhibitory concentration of the antimicrobials of veterinary interest.

Results: Ten strains were identified as *V. fluvialis* and one was identified as *V. lutrae*. The SE-AFLP analysis enabled the species differentiation with specific clustering of all *V. fluvialis* separately from the *V. lutrae* strain. Most strains presented growth in the maximum antibiotic concentration values tested for eight of the 10 analyzed antimicrobial classes.

Conclusions: The observed resistance pattern can represent a problem for veterinary and producers in the treatment of diseases associated *Vagococcus* spp. in swine production. *Vagococcus* species may also be a risk for pig industry workers. The data described here will be of great value in further understanding the behavior of this pathogen in animal production.

Key words: *Vagococcus lutrae*; *Vagococcus fluvialis*; MIC; SE-AFLP; Biochemical profile.

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Introduction

Vagococcus spp. is a Gram-positive, catalase-negative coccus that belongs to the family *Enterococcaceae* [1]. Phenotypically, this bacterium presents hemolytic colonies in blood agar incubated at 37ºC between 24 and 48 hours. These characteristics are similar to other systemic pathogens commonly associated with animal diseases [2,3].

Thirteen *Vagococcus* species have been described in a wide range of aquatic, terrestrial and insect species, and even in animal products [4-15]. Among them, the most frequently identified species and the one of major importance in domestic animals is *Vagococcus fluvialis*, originally described in chick feces and river water [1]. Another important species is *Vagococcus lutrae*, first isolated in an otter (*Lutra lutra*) [8]. These two species have already been associated with different infections in animal species and in humans [1, 2, 8, 16-19].

In swine, there are few reports of *V. fluvialis* as disease causative. Teixeira *et al.* [19] isolated some strains from different body organs, suggesting that this agent may play a role in systemic and opportunistic infections in pigs. In contrast, *V. lutrae* has not yet been described as a disease-causing bacterium in swine, but has already been isolated from humans, evidencing its zoonotic potential [16].

Here we report the isolation and identification of *V. fluvialis* and, for the first time, *V. lutrae* from diseased pigs. Therefore, the objective of this work was to characterize strains of *Vagococcus* spp. isolated from diseased pigs by phenotypic and genotypic techniques,

combined with epidemiological data, which are still rare information for this genus in swine production.

Methodology

Bacterial strains

Eleven strains with morphological characteristics similar to *Streptococcus* spp. (small, pale, smooth and alpha-hemolytic colonies) were isolated between 2012 and 2017 from lung, joint, brain, and vaginal discharge samples of nine pigs from eight herds of four different states in Brazil (Paraná, Mato Grosso, Santa Catarina, and São Paulo). The clinical conditions observed in these animals were encephalitis, arthritis, pneumonia, or urinary tract infection. The isolates were plated on Columbia agar (Difco-BBL, Detroit, MI, USA) with 5% sheep blood and incubated for 24 h at 37°C. Isolated colonies were inoculated in Brain-Heat Infusion (BHI - Difco-BBL, Detroit, MI, USA) supplemented with 5% fetal bovine serum and incubated for 24 hours at 37 °C for further analysis.

Identification by MALDI-TOF MS

For the matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) identification, sample processing and analysis were performed as previously described [20]. Bacterial mass spectra in the range of 2–20 kDa were acquired using a Microflex™ mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) and α-cyano matrix (10 mg α-cyano-4-hydroxy-cinnamic acid ml−1 in 50% acetonitrile/ 2.5% trifluoroacetic acid; Bruker Daltonik GmbH). The identification was performed with software MALDI BioTyper™ 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 the standard Bruker interpretative criteria were applied; scores ≥ 2.0 were accepted for species assignment and scores ≥ 1.7 but ≤ 2.0 for genus identification.

Identification by biochemical profile

The VITEK™ 2 automated identification system (bioMérieux, Hazelwood, MO, USA) was used to confirm bacterial strain identifications and to describe their biochemical profile. The inoculum preparation was performed according to the manufacturer's instructions and VITEK™ 2 GP ID card was used for identification of Gram-positive bacteria. Species identification was obtained using the VITEK™ 2 library system.

Antimicrobial resistance profile by minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by broth microdilution technique using Sensititre™ Standard Susceptibility MIC Plate (TREK Diagnostic Systems/Thermo Fisher Scientific, Waltham, MA, USA) according to the standards defined in CLSI document VET01, fifth edition [21]. The inoculum was prepared with BHI broth supplemented with 5% fetal bovine serum and incubated at 37°C for 24 h. The culture was adjusted to a turbidity equal to the 0.5 McFarland standard solution confirmed by a spectrophotometer. This suspension was diluted in the order of 1:1000 in Mueller Hinton II broth, supplemented with 5% fetal bovine serum. From this final suspension, 50 µL were distributed into each well of the microplate and incubated at 37 ºC for 24 hours. The *Streptococcus pneumoniae* ATCC 49619 strain was used as quality control.

The minimum inhibitory concentrations (MIC) were assessed visually as the lowest concentration of antibiotics in the wells without growth (without button formation). As there is no breakpoint available for *Vagococcus* spp. in the CLSI documents, the antimicrobials resistance assessment is presented as MIC profiles with the discrimination of the respective MIC values distribution.

DNA extraction and partial sequencing of 16S rRNA gene

Genomic DNA was extracted according to the protocol described by Boom *et al.* [22], with previous enzymatic digestion with lysozyme (100 mg) and proteinase K (20 mg) at 37ºC for 60 minutes. Partial amplification of the 16S rRNA gene was performed following the protocol and primers described by Twomey *et al.* [23].

The amplicons were purified using Illustra GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare) and sequenced by the Human Genome Research Center (University of São Paulo, Brazil). The obtained sequences were edited with the BioEdit Sequence Alignment Editor 7.2.6 software [24] and aligned by the ClustalW application [25] with the sequences available in the GenBank RefSeq database (National Center for Biotechnology Information; NCBI). Phylogenetic analysis was performed in the Mega 7.0.26 software [26] using the maximum-likelihood method and 500 bootstrap replicates for branch support statistical inference. The sequences

obtained from this study were deposited in GenBank under accession numbers MG098233 - MG098243.

Molecular genotyping by SE-AFLP

All strains were genotyped by Single-Enzyme Amplified Fragment Length Polymorphism (SE-AFLP) using the *Hind*III enzyme (New England Biolabs, Beverly, MA, USA) according to McLauchlin *et al.* [27] protocol. Electrophoresis was performed using a 2% agarose gel at 90 V for 4 hours. The amplified products were stained with BlueGreen™ (LGC Biotecnologia) and compared to 100 bp DNA ladder (New England Biolabs).

The Bionumerics 7.6 (Applied Maths) software was used for cluster analysis. A dendrogram was generated using the Dice coefficient and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The cut-off point of 90% of genetic similarity was applied to determine and analyze the obtained clusters [28].

Results

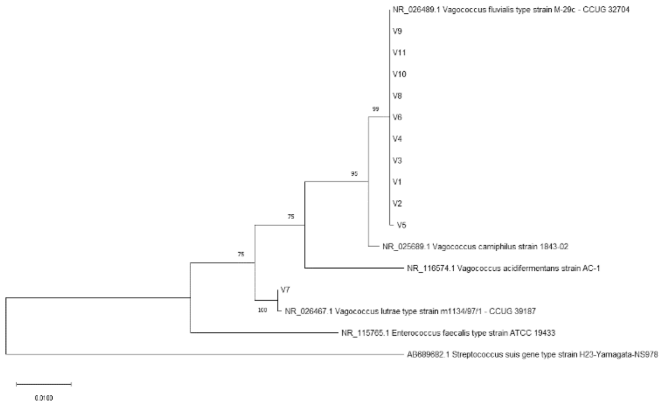
Bacterial identification

All studied strains were identified by MALDI-TOF MS, with score higher than 2.00, giving reliable species identification. Among studied strains, 10 were identified as *V. fluvialis* (V1 to V6 and V8 to V11) and one strain was identified as *V. lutrae* (V7). The identification was confirmed by VITEK™ 2 automated identification system and 10 strains were identified as *V. fluvialis* with more than 98% confidence. The V7 strain was not identified as *V. lutrae* by the VITEK™ system. However, the partial sequencing of the 16S rRNA gene was able to confirm both *V. fluvialis* and *V. lutrae* species (Figure 1).

Genotyping by single-enzyme amplified fragments length polymorphism (SE-AFLP)

The SE-AFLP fingerprint technique was able to identify seven different profiles (A1 – A7) with more than 90% genetic similarity. The largest genotypic profile identified was A1 formed by three strains, followed by A4 and A6 with two strains each, while the remaining profiles comprised only one strain each. The *V. fluvialis* strains presented high genetic heterogeneity – strains from the same animals (animals 4 and 8) did not cluster in the same genotypic profile, respectively. Furthermore, no associations between genotypes and epidemiological data, such as a geographic area, isolation site, year, and herd, were identified. However, at a level of 75% genetic similarity, the species

Figure 1. Phylogenetic tree based on the 16S rRNA nucleotide sequences for *Vagococcus* species confirmation.



differentiation was enabled with specific clustering of all *V. fluvialis* strains, and the *V. lutrae* strain separately.

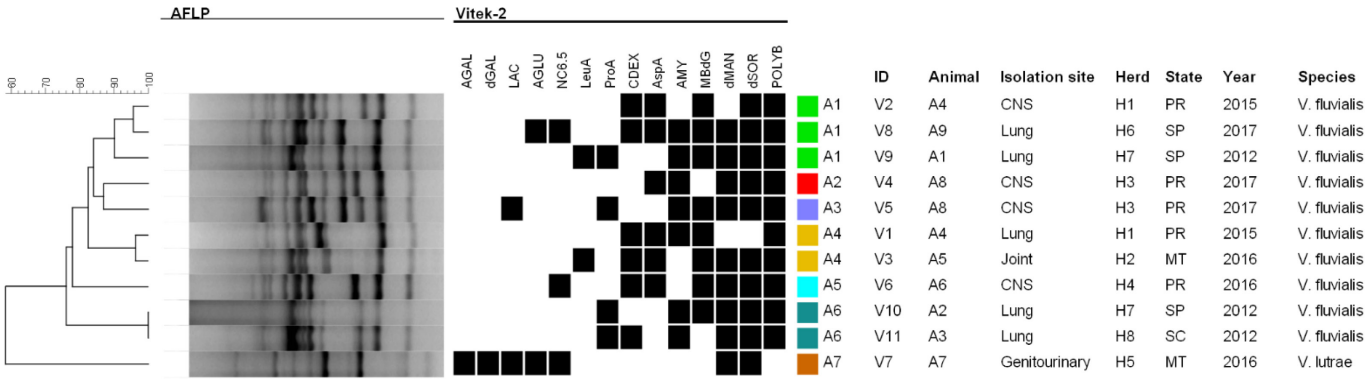
Biochemical profile

The biochemical profiles were identified by VITEK™ 2 for all isolates studied. The reactions for bacitracin resistance, D-maltose, D-mannose, D-ribose, D-trehalose, N-acetyl-D-glucosamine, novobiocin resistance, O/129-resistance, optochin resistance, L-pyrrolidonyl arylamidase, salicin and tyrosine arylamidase were positive among all studied strains, while reactions for arginine dihydrolase 1, arginine dihydrolase 2, alanine arylamidase, alpha-mannosidase, alanine-phenylalanine-proline arylamidase, beta-galactosidase, beta-galactopyranoside, beta–glucuronidase, D-raffinose, D-xylose, L- lactate alkalinization, phosphatase, phosphatidylinositol phospholipase C, pullulan, saccharose/sucrose, and urease were negative. This suggests that these

Table 1. Biochemical reactions that presented variation among *V. fluvialis* strains from swine.

Characteristic	Abbreviation	*V. fluvialis* strains with positive reaction % (N)
D-sorbitol	dSOR	10 (1/10)
D-manitol	dMAN	20 (2/10)
Methyl-b-d-glucopyranoside l	MBdG	20 (2/10)
D-amygdalin	AMY	30 (3/10)
L-aspartate arylamidase	AspA	40 (4/10)
Cyclodextrin	CDEX	40 (4/10)
L-proline arylamides	ProA	60 (6/10)
Leucine arylamidase	LeuA	80 (8/10)
Growth in 6.5% NaCl	NC 6.5	80 (8/10)
Alpha-glucosidase	AGLU	90 (9/10)
Lactose	LAC	90 (9/10)

Figure 2. Dendrogram based on SE-AFLP patterns of porcine *Vagococcus* spp. strains and their relationship with biochemical profiles.



CNS: Central Nervous System. PR: Paraná; SP: São Paulo; SC: Santa Catarina; MT: Mato Grosso. AGAL: alpha-*galactosidase*, AGLU: alpha-glucosidase, AMY: D-amygdalin, AspA: L-aspartate arylamidase, CDEX: cyclodextrin, dGAL: D-galactose, dMAN: D-mannitol, dSOR: D-sorbitol, LAC: lactose, LeuA: leucine arylamidase, MBdG: methyl-b-d-glucopyranoside, NC 6.5/NaCl 6.5%: Growth in 6.5% NaCl, POLYB: polymyxin B resistance, ProA: L-proline arylamidase.

biochemical reactions do not vary among *V. fluvialis* and *V. lutrae* species. In contrast, the reactions that showed variability within the *V. fluvialis* species are presented in Table 1. The *V. lutrae* strain was not directly identified by the VITEK™ system because this species is not indexed in the VITEK™ 2 library system. Nevertheless, the biochemical profile identified for *V. lutrae* shows a remarkable difference when compared to *V. fluvialis*, differentiating in positive reactions for alpha-galactosidase and D-galactose, and negative for D-amygdalin, L-aspartate arylamidase, cyclodextrin, leucine arylamidase, polymyxin B resistance, L-proline arylamidase, and methyl-b-d-glucopyranoside (Figure 2).

Antimicrobial susceptibility

Regarding the MIC profiles, *V. fluvialis* tended to present maximum values tested on plate for tiamulin, chlortetracycline, oxytetracycline, danofloxacin, sulfadimethoxine, tylosin tartrate, tulathromycin, tilmicosin, clindamycin, and enrofloxacin. *V. lutrae* strain presented growth in the maximum values of the antimicrobial concentrations tested for eight of the 10 analyzed classes. The only antimicrobials that inhibited

Table 2. Distribution of MIC values (mg/mL) identified among porcine *Vagococcus* spp. strains.

Antimicrobial	MIC Range (mg/mL)	Strains										
		V1	V2	V3	V4	V5	V6	V8	V9	V10	V11	V7*
Ceftiofur	0.25 - 8	4	1	> 8	4	8	> 8	2	> 8	> 8	> 8	≤ 0.25
Penicillin	0.12 - 8	1	0.5	8	2	1	8	0.5	8	8	2	≤ 0.12
Ampicillin	0.25 - 16	0.5	0.5	2	0.5	0.5	4	1	1	0.5	1	≤ 0.25
Gentamycin	1.0 - 16	2	2	2	**2**	4	4	2	2	2	4	> 16
Neomycin	4.0 - 32	32	> 32	> 32	> 32	> 32	> 32	32	16	16	> 32	> 32
Spectinomycin	8.0 - 64	16	> 64	32	> 64	> 64	> 64	> 64	16	32	> 64	> 64
Florfenicol	0.25 - 8	2	> 8	> 8	> 8	8	4	>8	4	4	> 8	> 8
Oxytetracycline	0.5 - 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8
Clortetracycline	0.5 - 8	4	>8	>8	>8	>8	>8	>8	>8	> 8	> 8	> 8
Enrofloxacyn	0.12 - 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2
Danofloxacin	0.12 - 1	> 1	> 1	>1	> 1	> 1	> 1	> 1	> 1	> 1	> 1	0.25
Tylosin	0.5 - 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	8	2	> 32	> 32
Tulathromycin	1.0 - 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	16	16	> 64	> 64
Tilmicosin	4.0 - 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	64	> 64	> 64
Clindamycin	0.25 - 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16
Tiamulin	0.5 - 32	> 32	> 32	> 32	> 32	> 32	>32	>32	> 32	> 32	>32	> 32
Sulfadimethoxine	256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
Cotrimoxazole	2/38	≤ 2/38	≤ 2/38	≤ 2/38	> 2/38	> 2/38	> 2/38	> 2/38	> 2/38	≤ 2/38	> 2/38	> 2/38

V7* *Vagococcus lutrae* strain.

V. lutrae growth were the beta-lactams and danofloxacin (Table 2).

Discussion

The results showed a high variability of the genotypic and phenotypic characteristics of the porcine *Vagococcus* spp. strains. Our results of SE-AFLP genotyping found no association between strain genotypes and epidemiological data. Teixeira *et al.* [19] used fingerprint techniques, such as Pulsed Field Gel Electrophoresis (PFGE) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), to identify profiles of different *Vagococcus* species. The SDS-PAGE enabled the differentiation of *V. fluvialis* from *Vagococcus salmoninarum* species at 80% genetic similarity; the authors could also differentiate these two species by PFGE profiles. In our study, the SE-AFLP technique also enabled the differentiation of the *V. fluvialis* and *V. lutrae* species, and further demonstrated high genetic heterogeneity among *V. fluvialis* strains. We also observed genetic heterogeneity among strains isolated from the same geographic region, which indicates the circulation of genetically different strains of the same species within a specific area.

The genetic differences can be in accordance with variations of some biochemical reactions identified among the *V. fluvialis* strains. Variations in the biochemical tests adonitol, L-arabinose, salicin and sucrose were described in strains of *V. fluvialis* isolated from a mosquito (*Culex quinquefasciatus*) compared to other strains of the same species [29]. Regarding the biochemical profile obtained for *V. lutrae*, the reaction for alpha-galactosidase was negative, which differs from the previous report, in which the reaction for both alpha- and beta-galactosidase were positive for *V. lutrae* and negative for *V. fluvialis* [8].

The growth of *V. fluvialis* strains in the highest concentrations tested for several of the studied antimicrobials may suggest potential resistance to these chemotherapeutic agents. Previously, data using disk diffusion technique for a *V. fluvialis* strain showed that, of the 18 antimicrobials tested, the strain was considered resistant to kanamycin, nafcillin, norfloxacin, clindamycin and nalidixic acid [28]. In another study, Teixeira *et al.* [19] also described MIC data for this species, which were described as resistant to clindamycin, lomefloxacin and ofloxacin.

More recent data on bacterial endocarditis in humans have shown that *Streptococcus viridans* and *Enterococcus* were the bacteria most frequently associated with these cases, but a third group called rare Gram-positive coccus was also identified, in which *V. fluvialis* is included, evidencing its zoonotic importance [18]. The decrease in the sensitivity for several of the tested antimicrobials in the present study may increase the risk in case of zoonotic infection. Garcia *et al.* [16] described antimicrobial susceptibility data from a human case of *V. lutrae* infection, and susceptibility were reported for amoxicillin, ceftriaxone, gentamicin, erythromycin, rifampicin, clindamycin, doxycycline, and vancomycin; however, the technique used to obtain such results was not clearly described in the study. In contrast, the results obtained here for *V. lutrae* strain demonstrate low susceptibility for several of these antibiotics. Considering these 11 strains, both *Vagococcus* species were not inhibited at the highest concentrations of at least seven antimicrobials classes tested, indicating a multi-resistant profile.

Clinical signs and disease described here and associated with *Vagococcus* species in swine, are not commonly related to this agent, and the occurrence of these species may be underestimated due to the misidentification in commercial laboratories. Thus, when the clinician encounters these cases in a herd, there may not be a good treatment response due to the antimicrobial resistance profile observed in these strains, increasing animal mortality and losses to the producers.

Conclusions

The data obtained show that *V. fluvialis* and *V. lutrae* species may represent a risk, not only for swine production, but also for pig industry workers due to the zoonotic potential of *Vagococcus* spp. All these data are of great help in further understanding the epidemiologic behavior of these neglected pathogens in swine production systems.

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Corresponding author

Andrea Micke Moreno, MV, MSc, PhD.
Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Science, University of São Paulo. Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508-270, São Paulo, SP. Brazil
Phone + 5502111 3091-1377
Fax: +5502111 3091-7928
E-mail: morenoam@usp.br

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