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Phylogenetic study and comparison of different TbpB obtained from *Glaesserella parasuis* present in Spanish clinical isolates

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ABSTRACT

Glaesserella parasuis (Gp) is the etiological agent of Glässer's disease (GD), which causes important economic losses for the pig intensive production worldwide. This organism uses a smart protein-based receptor to acquire specifically iron from the porcine transferrin. This surface receptor consists of transferrin-binding protein A (TbpA) and transferrin-binding protein B (TbpB). TbpB has been considered the most promising antigen to formulate a based-protein vaccine with broad-spectrum of protection against GD. The purpose of our study was to determine the capsular diversity of *Gp* clinical isolates collected in different Spanish regions between 2018 and 2021. A total of 68 *Gp* isolates were recovered from porcine respiratory or systemic samples. A species-specific PCR based on *tbpA* gene, followed by multiplex PCR for typing *Gp* isolates were performed. Serovars 5, 10, 2, 4 and 1 were the most prevalent and involved almost 84% of isolates. TbpB amino acid sequences from 59 of these isolates were analyzed, and a total of ten clades could be established. All of them showed a wide diversity with respect to capsular type, anatomical isolation site and geographical origin, with minor exceptions. Regardless of the serovars, the *in silico* analysis of TbpB sequences revealed that a vaccine based on a TbpB recombinant protein could potentially prevent Glässer's disease outbreaks in Spain.

1. Introduction

Glaesserella parasuis (*Gp*) is a Gram-negative, rod-shaped, host-specific organism belonging to Pasteurellaceae family sometimes having surface filaments of variable length like fimbria which are lost after subculture. Domestic or wild pigs are the *Gp* unique and natural hosts. This bacterial species is dependent on blood clotting V factor (nicotinamide adenine dinucleotide) and its *in vitro* growth requires the presence of this compound in a ratio of 0.01–0.025% (Liu et al., 2016; Münch et al., 1992).

Two groups of strains can be differentiated pathologically: the virulent and the non-virulent ones; these latter can be found in the respiratory tract of healthy pigs. Virulent and moderate-virulent strains can cause a systemic infection (Glässer's disease, GD) characterized by polyarthritis, polyserositis and meningitis, leading to significant economic losses to the pig intensive industry due to mortality and the expensive treatment of sick animals (Guizzo et al., 2018). *Gp* is transmitted very early (first week) from the infected sows to their offsprings (Cerdà-Cuéllar et al., 2010) and GD outbreaks have mainly been observed in the nursery phase. Two events are strongly associated with development of this disease: (i) the maternal antibodies decrease to a level that is not able to control bacterial replication, and (ii) farms that house animals from multiple sources may have multiple *Gp* serovars, making it difficult to have all the specific IgGs against all potential capsular antigens (Frandoloso et al., 2020).

GD has been reported in all countries with pig industrial production, with a different geographical distribution of serovars (SVs). Some countries possess the circulation of a few different capsular types because of the biosafety measures and low frequency of importing live animals. For instance, the most prevalent in North America, Australia, and Japan are SVs 4, 5, and 13, with about 15% (or even more in the case of Japan) of non-typeable strains (Rapp-Gabrielson and Gabrielson,

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1992). In Brazil, GD is associated with at least eight different SVs, but nine potential new SVs based on the size of capsular locus *Gp* have been shown (Pires Espíndola et al., 2019); of all of them, SV4 was the most prevalent. On the other hand, high frequency of clinical strains belonging to SVs 2 and 5 was described in Spain (Rúbies et al., 1999).

Gp has an iron acquisition system based on a surface receptor which binds specifically porcine transferrin. This surface receptor is composed of transferrin binding proteins A (TbpA) and B (TbpB) (Martínez et al., 2010). TbpA is an integral outer membrane protein that acts as a gated channel, while TbpB is a surface-exposed lipoprotein which captures the porcine iron-loaded transferrin and then transfers it to TbpA protein to transport the iron across the outer membrane (Martínez et al., 2010; Yang et al., 2011). In addition, TbpA and TbpB have become potential candidates for the development of vaccines against human (Moraes et al., 2009) or animal (Frandoloso et al., 2011; Frandoloso et al., 2013; Frandoloso et al., 2015; Martínez et al., 2010) pathogens, due to the pivotal role of these molecules during the infection.

There is a consensus among veterinarians that vaccine prevention is the most promising strategy to control the infection produced by *Gp*. It is also known that the use of vaccines based on capsular antigens confers mostly homologous protection (Takahashi et al., 2001); however, the strategic use of the TbpB as a vaccine antigen has the potential to overcome this last limitation, as already demonstrated in different studies (Frandoloso et al., 2015; Frandoloso et al., 2020; Guizzo et al., 2018). TbpB can be clustered into three main groups that were independent of species and serovar (Curran et al., 2015). The cluster 3 includes mostly *Gp* strains and, as previously (Frandoloso et al., 2015; Guizzo et al., 2018), a vaccine based on the mutant $TbpB^{Y\ 167A}$ which is clustered in this group can provide protection against *Gp* SV5 (Nagasaki strain, TbpB cluster 3) and SV7 (174 strain, TbpB cluster 3). Therefore, this TbpB cluster can predict the profile of protection of a TbpB-based vaccine.

Moreover, for the elucidation of drug–target interaction and optimization of therapeutic outcome, comprehensive *in vitro* and *in vivo* investigations along with relevant computational studies are required (Heidarpour et al., 2011; Mohammadabadi and Mozafari, 2018). *In silico* study is one of the main methods to evaluate the activity of new drugs and bioactive agents by computational structure-based drug discovery (Alavi et al., 2022; Mohammadabadi et al., 2009).

Because of the importance of monitoring the *Gp* capsular type and the diversity of TbpB antigen among the clinical isolates associated with GD outbreaks in Spain, the aim of this study was (i) to update the *Gp* capsular type that are circulating nowadays in Spain and (ii) to know the diversity of TbpB in these Spanish isolates to predict the antigenic coverage of the recombinant TbpB-based vaccine.

2. Materials and methods

2.1. Collection of isolates

A total of 68 *Gp* isolates were obtained from samples sent by different pig farms located in Spain; concretely, in "Castilla y León" (45.6% of isolates), "Cataluña" (20.6%), "Extremadura" (13.2%), "Aragón" (10.3%), "Andalucía" (4.4%), "Murcia" (2.9%), "Galicia" and "Castilla-La Mancha" (1.5% each) regions between October 2018 and March 2021. Sterile swabs were taken from injured areas such as the lungs (72.1%) or systemic sources (20.6%) and were cultured on chocolate blood agar. The anatomical origin was unknown in 7.3% of samples. Morphologically suspected colonies were confirmed by means of PCR (de la Puente Redondo et al., 2003) and then frozen at −80 °C until later use.

1.1. PCR method for identification of Glaesserella parasuis

Once the DNA was extracted by boiling, the species identification was performed by means of a PCR for *tbpA* gene (de la Puente Redondo et al., 2003). The mixture for the PCR reaction consisted of 3 μL of extracted DNA, 1 U of Taq polymerase (Roche Diagnostic), 5 μL of 10× amplification PCR buffer, 1.5 μL of $MgCl_2$, 1 μL of each of the primers (20 μM), 0.5 μL of dNTPs (25 mM each), and nuclease-free water to a final volume of 50 μL. TbpA F (5' TGGTGGCTTCTATGGTCCAA 3′) (Frandoloso et al., 2020) and RTC 33 (5' AAGCTTGAAACTAAGGTACTCTAA 3′) (de la Puente Redondo et al., 2003) were used as primers. An isolate previously identified as *Gp* was used as positive control while nuclease-free water was used as negative control. The reaction was carried out in a thermocycler (Eppendorf Mastercycler® Gradient, Germany), with an initial treatment of preincubation of 5 min at 94 °C, followed by 35 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 50 °C and 1 min of extension at 72 °C). The final extension was performed for 10 min at 72 °C.

2.2. Multiplex PCR for molecular typing of Glaesserella parasuis

Once the isolates were confirmed, a multiplex PCR (Howell et al., 2015) was performed to identify the SV to which each isolate belonged to. For the individual SV-specific primers, a reference strain of each one was used as positive control, and nuclease-free water was selected as negative control. Amplification was performed in a thermocycler (Eppendorf Mastercycler® Gradient, Germany), with an initial treatment of 30 s at 94 °C, followed by 30 cycles of 3-step cycling comprising denaturation for 30 s at 94 °C, annealing at a temperature range between 52 and 64 °C of the gradient PCR for 30 s, 1 min at 68 °C for extension, and a final extension of 5 min at 68 °C.

2.3. Amplification of transferrin binding protein B (TbpB) gene

The mixture of the reaction consisted of the same reagents described above. TbpB F1 (5' ACTACTTTCCCTAAGCCTGT3') and TbpB RRTC 33 (5' CCGTGGAATATCAACATATCG3') (Curran et al., 2015) were used as primers. gDNA from *Gp* SV 5, Nagasaki strain, was used as positive control, and nuclease-free water was used as negative control. The reaction was carried out in a thermocycler (Eppendorf Mastercycler® Gradient, Germany) using the same conditions that for *tbpA* gene.

2.4. Sequencing

Genomic DNA (gDNA) was sequenced from a total of 59 isolates. PCR products were purified using the GeneJet Gel Extraction and DNA Cleanup Micro Kit (ThermoFisher, USA), and then, the DNA concentration was measured using Nanodrop™ 2000 (ThermoFisher, USA). DNA samples were later sequenced by the Sanger method. An intermediate primer was needed to obtain the whole nucleotide sequence of *tbpB* gene. The sequence for this primer (TbpB I) was 5' ATTACTACAGCAAGCTACAGCTACC 3′ (Curran et al., 2015).

Once the samples were sequenced, they were edited with the Chromas™ software, the contigs were performed with the Bioedit™ software, and they were collected in the same file. Alignment and phylogenetic analysis of amino acid sequences were carried out using Clustal W and Mega7™ (Molecular Evolutionary Genetics Analysis software). Statistical support of the tree structures was obtained by 100 bootstraps. All the positions with less than 95% site coverage were eliminated, that is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

3. Results and discussion

The 68 *Gp* isolates tested came from thirteen provinces belonging to eight regions located in the four cardinal points, representing the epidemiological situation of GD in Spain. Most of them (29.4%) belonged to SV 5, followed by SV 10 (17.6%), and thirdly by SVs 2 and 4 (13.2% each). SV 1 accounted for 10.3%, while SVs 3, 7, 8 and 12 involved less than 8% (Table 1). SV 5 was also the most prevalent in

Table 1
Serovars of *Glaesserella parasuis* according to the isolation source.

Serovar	Source of isolation (number and percentage)			
	Lungs	Systemic	Unknown	Total
1	3 (42.9)	3 (42.9)	1 (14.2)	7 (10.3%)
2	7 (77.8)	2 (22.2)	–	9 (13.2%)
3	3 (75.0)	1 (25.0)	–	4 (5.9%)
4	7 (77.8)	2 (22.2)	–	9 (13.2%)
5	15 (75.0)	3 (15.0)	2 (10.0)	20 (29.4%)
7	4 (80.0)	–	1 (20.0)	5 (7.4%)
8	1 (100.0)	–	–	1 (1.5%)
10	8 (66.7)	3 (25.0)	1 (8.3)	12 (17.6%)
12	1 (100.0)	–	–	1 (1.5%)
Total	49 (72.1)	14 (20.6)	5 (7.3)	68

other study carried out in Spain at the end of the 20th century (Rúbies et al., 1999), as well as in other Danish (Angen et al., 2007), Chinese (Ma et al., 2016; Zhao et al., 2018) or Vietnamese (Van et al., 2019) investigations. However, the prevalence of *Gp* in our study was completely different from that found in Brazil, where SV 4 was the most recovered from GD outbreaks in this American country (Pires Espíndola et al., 2019), the same as it was in China (Jia et al., 2017), Malaysia (Lee et al., 2019) or in other European countries like Italy (Luppi et al., 2013). Like ours, the majority of *Gp* isolates recovered in the east of Europe included SVs 2 and 5 (Docic and Bilkei, 2004). Surprisingly, no non-typable isolates were found using multiplex PCR, unlike previous reports in which noticeable rates of non-typable *Gp* were detected in other countries (Angen et al., 2007; Docic and Bilkei, 2004; Pires Espíndola et al., 2019) and even in Spain (de la Puente Redondo et al., 2003; Rúbies et al., 1999), using molecular (Angen et al., 2007; de la Puente Redondo et al., 2003; Docic and Bilkei, 2004; Oliveira et al., 2003) or serological (Docic and Bilkei, 2004; Oliveira et al., 2003; Rúbies et al., 1999) methods.

In order to analyze the diversity of the TbpB, we conducted the sequencing of *tbpB* whole gene from a total of 68 *Gp* clinical isolates. However, due to failures in the sequencing process as well as to certain mutations in some of these isolates, nine of them could not be correctly sequenced (Tavaré, 1986).

A phylogenetic tree was then constructed using the Neighbor-joining clustering method (Fig. 1) to achieve a clear idea of the relationship of TbpB amino acid sequences from the 59 isolates. Ten clades with bootstrap support were shown: clade 4 was the largest of all, with 12 isolates (20.3%); clades 1 and 3 contained 11 isolates each (18.6%); clade 6 included eight isolates (13.6%); clade 2 harboured five isolates (8.5%); clade 9 had four isolates (6.8%); clade 8 contained three isolates (5.1%); clade 5 and 10 included two isolates each (3.4%) and clade 7 added one isolate (1.7%).

Clades 1 and 3 were the most heterogeneous of all concerning serovar distribution because six of the nine serovars were represented in each one of them, and they did not stand next to each other. Clade 6 was also diverse because its eight *Gp* isolates belonged to five serovars, the same as clade 2, with five members distributed among three serovars. However, although clade 4 was the largest, it was much more homogeneous because six of its members (50%) belonged to SV 2 and they stood next to each other. The four *Gp* isolates in clade 9 belonged to two serovars and, finally, clades 5, 7, 8 and 10 kept all SV 5 isolates.

A far evolutionary relationship could be revealed according to the geographical location of isolates. As much, that four (33.3%) of the isolates recovered from "Segovia" province ("Castilla y León") belonged to clade 4, that other three (27.3%) were linked to clade 1, or that four (36.4%) from those recovered in "Lérida" province ("Cataluña") belonged to clade 3, to mention some proximity. On the other hand, phylogenetic analysis of TbpB amino acid sequences segregated independently of the anatomical origin from which these *Gp* isolates were recovered for clades 1–4 and 8–10, but nevertheless, all members included in clades 5, 6 and 8 had a lung origin. This finding indicated that clustering was only partially determined by the source from that the

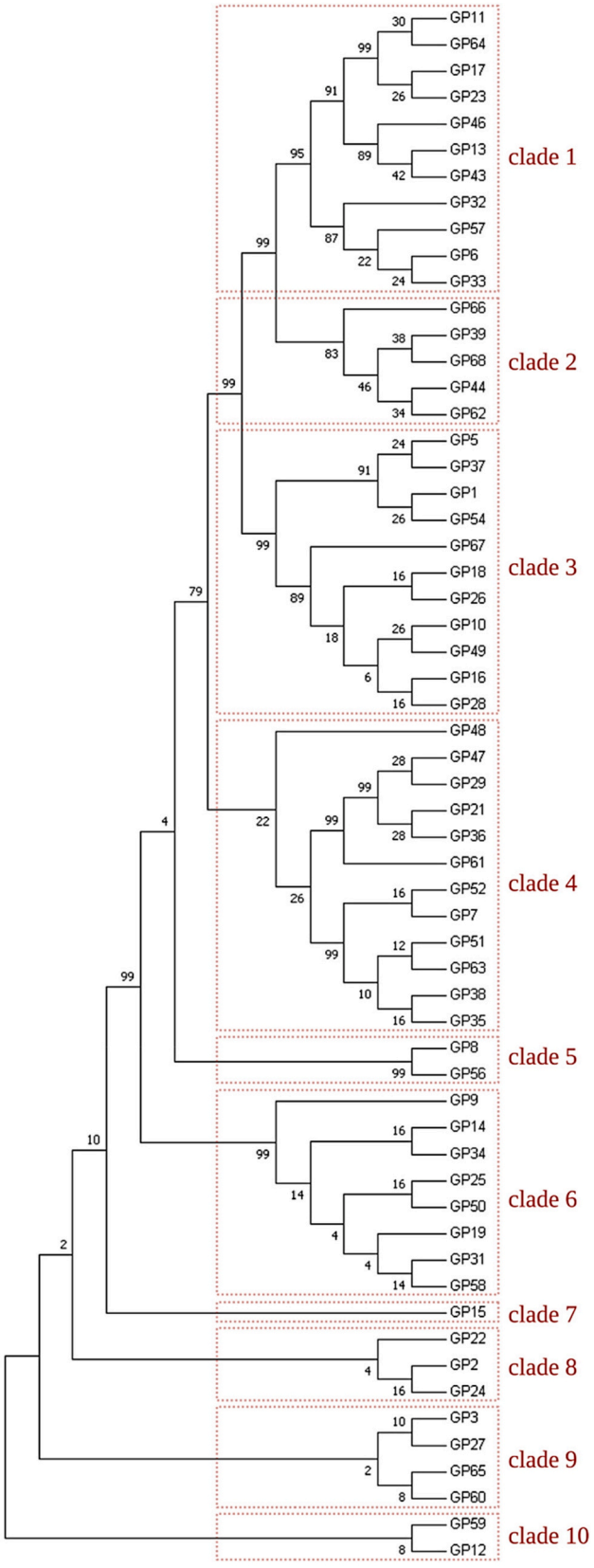

Fig. 1. Phylogenetic tree of TbpB amino acid sequences from 59 Spanish clinical isolates of *Glaesserella parasuis*. Neighbor-joining clustering method. GP (followed by a number) is the reference of each of the isolates. Bootstrap values based on 1000 replicates are shown at branch nodes.

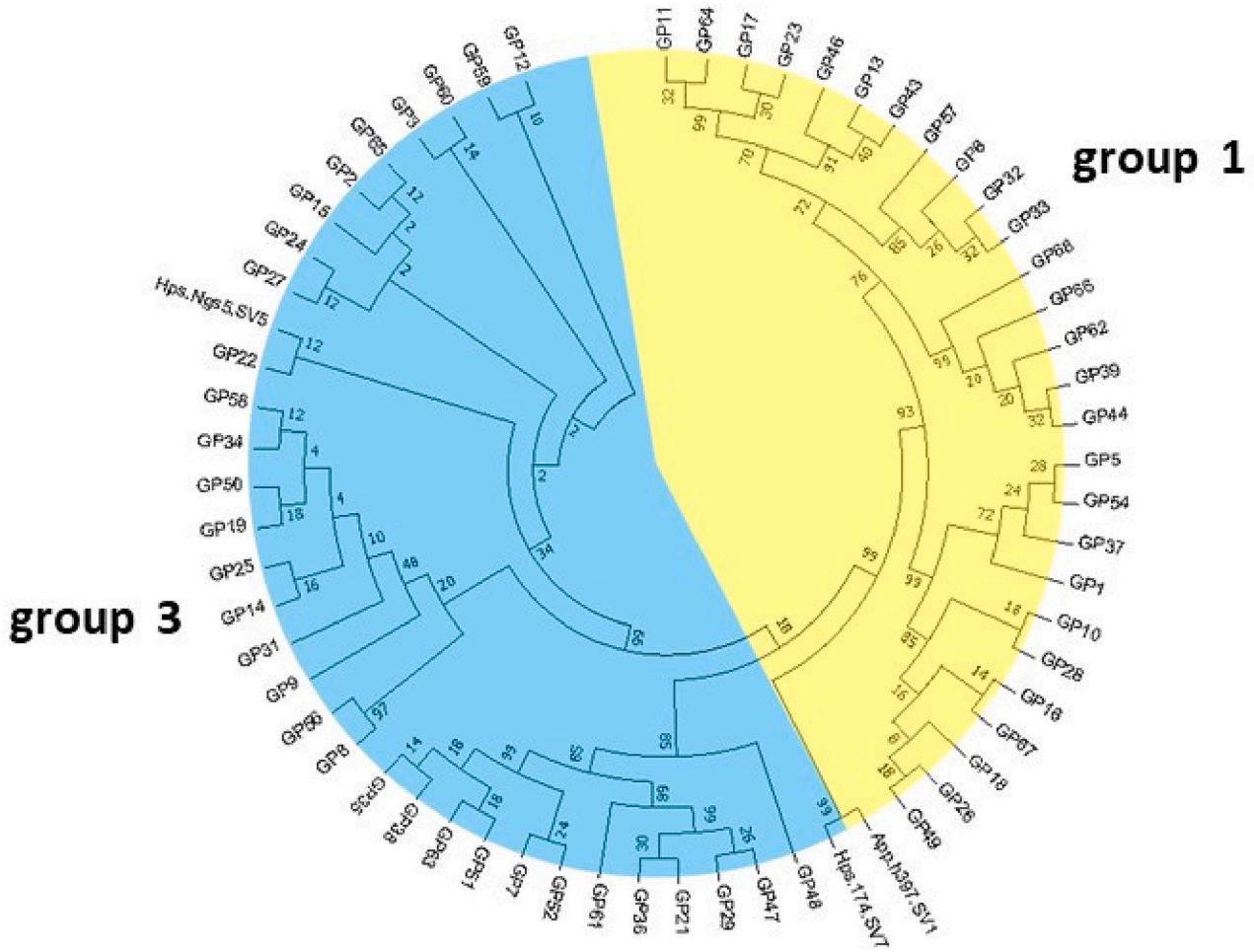

Fig. 2. Classification of the TbpB protein in relation to its antigenic group. A total of 59 Spanish TbpB amino acid sequences were aligned with one sequence belonged to TbpB group 1 (App.h397.SV1) and two sequences belonged to TbpB group 3 (Hps.Ngs5.SV5 and Hps.174.SV7) (Guizzo et al., 2018). The vaccine antigen TbpB^{Y167A} (Frandoloso et al., 2015), used to formulate protective vaccines against *Gp* (Frandoloso et al., 2015; Frandoloso et al., 2020; Guizzo et al., 2018) is derived from one *G. parasuis* strain belonging to TbpB group 3. The colors fit with the groups described by Guizzo et al. (Guizzo et al., 2018): yellow color belongs to TbpB group 1 and blue color belongs to TbpB group 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

isolates were recovered.

Therefore, these results show that the sequences of the 59 *Gp* clustered quite independently from geographical region or serovar. This fact suggests that a virulent isolate or serovar could alter its TbpB variant or acquire a different TbpB variant during a GD outbreak in a specified geographical region. In addition, our finding fully matches with those previously reported for other North American, Asian or Western European *Gp* isolates (Curran et al., 2015; Guizzo et al., 2018).

Next, a comparison with our 59 TbpB sequences and those from this same species or other Pasteurellaceae reported by Guizzo et al. (Guizzo et al., 2018), was performed. As illustrated in Fig. 2, 32 of Spanish clinical strains (54.2%) expressed TbpBs classified as belonging to group 3. Another 27 *Gp* strains (45.8%) were classified within group 1, and no isolates were included in group 2. Considering the protective capacity of the TbpB^{Y167A}-based vaccine, which confers *in vivo* protection against *Gp* isolates belonging to TbpB group 3 (Frandoloso et al., 2015; Frandoloso et al., 2020; Guizzo et al., 2018) and to TbpB group 1 (unpublished results). Our results suggest that this vaccine would have the real potential to control the infection of *Gp* isolates circulating in the Spanish swine herds. Even so, future studies are needed to demonstrate, under controlled pig infection model, whether TbpB^{Y167A}-based vaccine could prevent the development of Glässer's disease caused by Spanish *Gp* clinical isolates belonging to TbpB groups 1 and 3.

4. Conclusions

SV 5 was the most prevalent from the 68 *G. parasuis* isolates recovered in Spain between 2018 and 2021. A total of eight additional SVs were identified, and the sequencing analysis of TbpB protein showed that the use of the TbpB^{Y167A}-based vaccine might protect against the emergence of Glässer's disease produced by all clinical isolates assessed in the present study.

These results pave the way for future research, such as testing whether a TbpB-based vaccine could prevent the development of Glässer's disease caused by Spanish *Gp* clinical isolates belonging to different TbpB groups.

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CRediT authorship contribution statement

Alba González Fernández: Methodology, Investigation, Validation, Formal analysis, Writing – original draft. **César Bernardo Gutiérrez Martín:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Máximo Petrocchi Rilo:** Methodology, Investigation. **Esther Pérez Fernández:** Methodology, Investigation. **Rubén Miguélez Pérez:** Methodology, Investigation. **Rafael Frandoloso:** Conceptualization, Writing – review & editing. **Sonia Martínez Martínez:** Conceptualization, Validation, Writing – review & editing, Funding acquisition.

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