Detection of *Streptococcus suis* using the optimized real-time polymerase chain reaction protocol

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The article presents the results of studies on the detection of *Streptococcus suis* by real-time polymerase chain reaction. Isolation and species identification of the studied isolates of streptococci was carried out according to morphological, cultural, biochemical and biological properties by conventional methods. The study of cultural characteristics of growth was carried out using conventional bacteriological methods on the brain heart infusion broth (BHI) and BHI agar with the addition of 5% sheep blood (blood BHI agar). To confirm biochemical properties as a confirmatory method, API 20 STREP test kit (bioMerieux, France) was used. In addition, to differentiate *S. suis* from the non-pathogenic species of streptococci, the hemolysis test was used. As a result of the studies, it was found that the use of the real-time PCR (polymerase chain reaction) method makes it possible to detect *S. suis* in an amount of 1×10^4 genome copies in the sample. All described validation parameters for the qualitative detection of *S. suis* DNA by real-time PCR meet international requirements, which guarantees accurate and reliable results. In Ukraine only a diagnostic test kit for convential PCR has been developed for the detection of swine streptococcosis. This approach is more time consuming and complex in comparison with the real-time PCR approach. We recommend that diagnostic laboratories implement this method in their practice. This will increase the number of effective diagnostic tools available to veterinarians on pig farms when they order laboratory tests. The high analytical sensitivity limit of a test is an essential parameter when screening is the focus, and obtaining false negative results causes a risk of the development of infection process among pig populations within infected herds. Our study showed that microbiological diagnostic methods to determine morphological and cultural properties can identify *S. suis* at the genus level. Determination of biochemical properties using the API 20 STREP test kit can be used to identify *S. suis* 1 and 2 serotypes. The conventional method and real-time PCR have 100% specificity and can be used to identify *S. suis* of different serotypes. Real-time PCR is a 2 to 4 times more sensitive limit than conventional PCR depending on the serotype being studied, and can be used to more accurately identify streptococcal DNA. It was found that the use of the real-time PCR method makes it possible to detect *S. suis* in an amount of 1×10^4 copies of the genome in the sample. Additionally, it was found that all the studied validation parameters of the qualitative method for determining *S. suis* DNA by real-time PCR meet international requirements, which guarantees accurate and reliable results.

Keywords: pathogenic potential; zoonosis; biological material; sensitivity limit; PCR; local infections; epizootology; molecular research.

Introduction

Streptococcus suis is one porcine pathogen that is highly prevalent worldwide and causes economic damage due to reduced productivity in swine production. Streptococcosis of pigs is registered in the form of acute and subclinical manifestations of the disease, namely: meningitis, arthritis, damage to the respiratory system with endocarditis and dermatitis (Ouattara et al., 2020). The natural habitat of *S. suis* is the upper respiratory tract, including the tonsils and nasal cavity, and the digestive system of pigs (Kerdsin et al., 2012). A total of 35 serotypes of *S. suis* (1–34 and individual type 1/2) colonizing the body of pigs were identified and classified according to their capsular antigen. Serotypes 1–9 are most often isolated from animals with clinical symptoms, another 15.5% of strains may not be typical due to their variability. In pigs in Europe serotype 9 is most often isolated. *S. suis* type 2 is most often isolated from sick people, it is believed that it has the greatest zoonotic potential, which differs between different strains of this group. *S. suis* 2 type ST 1 most often causes human disease in Europe, Asia

and Australia (Wang et al., 2018; Hlebowicz et al., 2019). The pathogenetic and zoonotic potential of *S. suis* is due to the presence of various groups of virulence factors. The specific set of such factors is variable, differing in different types and strains. As a result, *S. suis* has a high ability to adapt to environmental influences, avoid the host's immune response and successfully colonize the target organism or initiate an infectious process (Wang et al., 2015; Werinder et al., 2020). *Streptococcus suis* accumulates in the tonsils of clinically ill and outwardly healthy pigs and is usually transmitted nasally or orally (Huang et al., 2021). Asymptomatic carriers (carrier animals) of *S. suis* are a source of infection for other pigs and are important in the transmission of this pathogen in herds. *Streptococcus suis* is resistant to various environmental conditions (Srinivasan et al., 2016; Bleuzé et al., 2021).

The pathogen can be transmitted by contact, or by aerogenic and alimentary routes between animals and humans. First, the colonization of the organism occurs, if the colonizing strain has the necessary pathogenic factors or the target organism is weakened, then the infectious process

begins (Tarasov et al., 2021). Local infections occur in the form of arthritis, endocarditis or pneumonia. If the local immune response is not sufficient to localize the lesion, the pathogen will continue to enter the bloodstream (Ferreira et al., 2018). This can lead to a generalized infection or cause meningitis when crossing the blood-brain barrier. At risk are all employees of pig farms as well as consumers of unprocessed meat products (Dutkiewicz et al., 2018; Sunaga et al., 2020). Due to the number of serotypes and genetic diversity of strains, the diagnosis of *S. suis* requires clear diagnostic criteria and the use of methods that will minimize the impact of the natural variability of the infectious agent. Based on morphological and cultural properties, it is quite simple to determine the affinity of the pathogen to the genus *Streptococcus* spp. They need to be studied to identify a pure culture and further establish the pathogenicity, lethality of the pathogen and the profile of antibiotic resistance (Gottschalk et al., 1991; Vaillancourt et al., 2015). However, enzymatic properties are variable within the genus and species, so microbiological methods of differentiation can be used only in combination with other types of methods. A commercial kit for microbiological identification of *S. suis* 1 and 2 type API 20 STREP is available. Identification of an agent by the MALDI-TOF MS method requires the development of databases for accurate identification at the level of species and serotypes. Currently developed serological diagnostic methods are aimed at detecting only 2 serotypes of the pathogen that have the greatest zoonotic potential (Yi et al., 2020; Jiang et al., 2022).

Molecular genetic methods can accurately identify the pathogen. There are schemes that determine the pathogenic potential and role of the agent in the infectious process based on the sequenced DNA of the pathogen. But such methods are expensive and difficult to interpret, which complicates their use by a veterinarian. Different PCR (polymerase chain reaction) methods are easy to interpret and cost-effective. There are various modifications of PCR aimed at fragments of genes specific to *S. suis*. Methods of conventional PCR and multiplex PCR are aimed at determining the genes recN, 16S rRNA, gdh. Multiplex PCR for typing a number of serotypes is directed to areas of the cps gene. There is multiplex PCR which aims to identify fragments of genes associated with pathogenicity. Quantitative PCR is directed to the fbpS and 16S rRNA genes (Werinder et al., 2021; Xu et al., 2021; Liang et al., 2022).

After analyzing the publication of various authors, we suggest that the PCR method may be the most effective for identifying the pathogen *S. suis* and differentiating it from other *Streptococcus* spp. microorganisms. PCR is a fast and high-precision research technique used to detect the pathogen in samples taken from infected or healthy pigs, or even from sick people for clinical diagnosis or epidemiological studies (Goyette-Desjardins et al., 2014; Xia et al., 2018). Real-time PCR has shown high efficacy in detecting the causative agent of streptococcus not only at different stages of the disease but also in asymptomatic patients. Therefore, the obtained data show an extremely high expediency of real-time PCR for the identification of *S. suis* (Pérez-Sancho et al., 2015; Segura et al., 2017).

Establishing a rationale for the choice of method and optimizing and validating the protocol of detection of *S. suis* by setting a qualitative real-time polymerase chain reaction in accordance with international standards is important for improving the diagnosis of the disease in veterinary laboratories. This was the aim of our study. All described validation parameters for the qualitative detection of *S. suis* DNA by real-time PCR meet international requirements (ISO 17025), which guarantees accurate and reliable results.

Materials and methods

For the tests we used strains of *S. suis* which are stored in the museum of the Institute of Veterinary Medicine of the National Academy of Sciences of Ukraine. A reference culture of *S. suis* NCTC 10234 was used as a positive control. *Staphylococcus aureus*, *Micrococcus luteus*, *Enterococcus faecalis*, *Streptococcus agalactiae* from the Institute of Veterinary Medicine the National Academy of Agrarian Sciences of Ukraine (IVM NAAS) collection were tested for control of reaction specificity. 9 strains of *S. suis* in serotypes 1, 2, 1/2, 5, 7, 10, 14, were isolated from pathological material from pigs with clinical signs acute streptococcosis and confirmed by the conventional PCR method (Tarasov et al., 2021).

The strains were stored in semi-liquid nutrient agar media (HiMedia M001-100G Nutrient Agar, 4 g/L water, manufacturer HiMedia Laboratories, India) in a refrigerator at 8 °C. To prepare the research, the culture was subcultured on nutrient agar media (HiMedia M001-100G Nutrient Agar, manufacturer HiMedia Laboratories, India) with the addition of 10% horse serum (Donor Horse Serum, manufacturer Ukrmediasnab, Ukraine) and 2% glucose. Morphological and cultural properties of the museum strain and field isolates were typical of *S. suis*. After growing on BHI agar, it was found that small, smooth, transparent, white S-shaped colonies formed in 24–48 hours after incubation for 72–96 hours.

Isolation and species identification of the studied isolates of streptococci was carried out according to morphological, cultural, biochemical and biological properties by conventional methods. The study of cultural characteristics of growth was carried out using conventional bacteriological methods on the brain heart infusion agar (BHI agar) with the addition of 5% sheep blood (blood BHI agar). To confirm biochemical properties, as a confirmatory method, we used API 20 STREP test kit (bioMerieux, France). In addition, to differentiate *S. suis* from the non-pathogenic species of streptococci, we conducted the hemolysis test. To isolate DNA 2-3 typical colonies were selected from the nutrient agar surface, and diluted with sterile saline solution to 0.5 McFarland standard (approximately 1×10^8 cells/mL). DNA was extracted with lysing buffer, which consisted of 20 mL of 1 M TrisHCl (pH 8.5), 100 µL of Tween 20, 48 mg of proteinase K and 32 mL of water for molecular genetic studies. Material – colonies of the microorganism from Petri dishes with BHI agar were resuspended in PBS (phosphate-buffered saline) and were inactivated at a temperature of 96 °C for 300 seconds. All PCR reactions were carried out using isolated DNA in an amount of 20–30 ng/µL (nanogram on microliter) measured by Nanodrop spectrophotometer (TermoFisher, USA).

Primer set for real-time PCR was used according to recommendation Srinivasan et al. (2016) which flank the nucleotide sequence of fbpS gene of *S. suis*: 5′-TCC RAT RCT GCT CTG CCA TT-3′ and 5′-ATGA TAG TAG AAG TCC AGC ARA CT-3′ (GenBank No. CP003993, manufacturer Thermo Fisher Scientific, USA), amplicon size 114 bp (base pairs). Probe for detection of amplification was tagged with a FAM-AA TAG CCC "T"GA AAA MCA GCC ACW YTT TGA RA-6SpC; "T" = BHQ1 (manufacturer Lumiprobe GmbH) and a BHQ1. The reaction mixture contained PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 0.25 mM of each dNTP, 2.5 mM MgCl₂, 10 pM of each primer, 5 pM of probes, and 0.5 units of Taq-DNA polymerase (Thermo Fisher, USA). Amplification reaction conditions are shown in Table 1. For the research, a Rotor-Gene 6000Q real-time amplifier (QIAGEN Hilden, Germany) was used.

Table 1
Amplification program for real-time PCR *S. suis* detection

Stage	Temperature, °C	Time, s	Cycle
Activation of polymerase	95	360	1
Denaturation/annealing/extension	95/55/72	20/20/20	35
Final extension	72	300	1

A positive reaction was considered in the case of the presence of a specific fragment of appropriate size in the test sample and the positive control, and the absence of the reaction product in the negative control. Optimization of real-time PCR was performed by changing the annealing temperature of the primer. According to the polymerase passport, the annealing temperature was taken to be 5 °C below the average melting point of the primers (Tm). Sequential PCR reactions with variable annealing temperature were then performed. The following annealing temperatures (Ta) of 50, 53, 55, 57 and 60 °C were used in the study. The optimum temperature at which the reaction product was present and the lowest SD (Standard deviation) and CV (coefficient of variation) was considered. We compared the calculated coefficient of variation (CV, %) of the Ct (cycle threshold) values for a series of sample studies at the same time with an acceptable value of the coefficient of variation (CVv, %), which was obtained from the validated standard deviation value for this method (SDv, % for the method not more than 0.5). The analytical sensitivity limit of conventional PCR and real-time PCR was compared. For conventional PCR, consecutive decimal dilutions of the culture with a known number of CFU (colony-forming unit) were performed. The initial in an amount was 0.5 according to the McFarland standard (1×10^8),

5 consecutive dilutions were performed. The first dilution for real-time PCR analytical sensitivity limit was the last dilution of conventional PCR to give the reaction product.

For the conventional PCR, we used primers 5′-TTC TGC AGC GTA TTC TGT CAA ACG-3′ and 5′-TGT TCC ATG GAC AGA TAA AGA TGG-3′, that flank a 695 bp fragment of the glutamate dehydrogenase gene (gdh). Amplification conditions are described in Table 2.

Table 2
Amplification program for conventional PCR *S. suis* detection

Stage	Temperature, ℃	Time, s	Cycle
Activation of polymerase	95	600	1
Denaturation/annealing/extension	62/95/62	20/20/20	35
Final extension	72	300	1

The amplicons were detected by electrophoresis using 10 μL of the amplified product in a 2% agarose gel with ethidium bromide gel stain (Invitrogen®, USA) as dye. A 100 bp (base pairs) molecular marker (Invitrogen®, USA) and a positive control for *S. agalactiae* were used to evaluate the PCR products. The fragment with presence of 695 bp amplicon were considered to be positive for *S. suis*. Deionized water was used as a negative control for DNA isolation and preparation of the reaction mixture. The presence of reaction products in front of wells with negative controls indicated the presence of contamination. A positive reaction was considered in the presence of a specific fragment of appropriate size in the test sample and a positive control, and the absence of the reaction product in the negative control.

The results of experimental studies were processed by traditional statistical methods using the R software package for statistical computing (R version 3.4.2, R Core Team, 2016). Results were considered statistically significant when $P < 0.05$. Standard deviation (SD) and coefficient variation (CV) indicators were determined in accordance with the requirements of the ISO standard.

Results

Microscopy revealed gram-positive, cocci-like bacteria located in a chain or in pairs. The presence of α-hemolysis zones was detected on blood agar. When determining the enzymatic properties using the API 20 STREP test kit, we found that they differ between serotypes. Serotypes 1 and 2 were accurately identified, and other streptococci studied had variable characteristics. Using this test kit, it was possible to identify control strains of *E. faecalis* and *S. agalactiae*. It is not possible to determine *S. aureus* and *M. luteus* with this test kit.

In the study of samples by conventional PCR, we found that the control strain and field isolates had a corresponding reaction product size of 695 bp. Nonspecific fragments were absent. In negative controls, the reaction product was absent. The specificity of the method is 100%. Examining the sensitivity limit of the conventional PCR method, we found that at an amount of bacteria in solution (CFU/cm³) of 1×10^8 the reaction product was formed in 10 samples from 10 test samples (100% analytical sensitivity), in an amount of 1×10^7 in 9 samples from 10 test samples (90%) test samples, in an amount of 1×10^6 in 8 samples from 10 test samples (80%), in an amount of 1×10^5 in 3 samples from 10 test samples (30%). At a lower amount of reaction products were not formed. Optimi-

zation of the real-time PCR method was performed by setting successive reactions with a change in the annealing temperature of the primers. DNA was isolated from a bacterial suspension of the museum strain and suspensions of field isolates diluted to 0.5 according to the McFarland standard (1×10^8 CFU). Amplification was performed with annealing temperatures (Ta) of 50, 53, 55, 57 and 60 ℃ (Table 3). For annealing temperatures of 50 ℃ (SD 4.46%) and 60 ℃ (SD 0.63%), the indicator SD < 0.5 is out of range of reliability (Fig. 1).
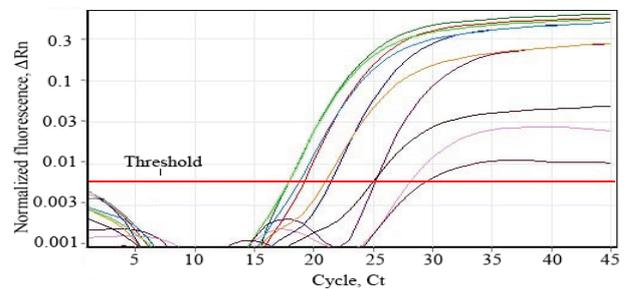

Fig. 1. Results of *S. suis* DNA amplification of studied samples using annealing temperature of 50 ℃, obtained using the FAM/Green channel: ΔRn – relative units of fluorescence which are used to build a graph; Ct – cycle threshold

Temperatures of 53 ℃ (SD 0.50%), 55 ℃ (SD 0.26%) and 57 ℃ (SD 0.34%) meet the requirements for the optimized protocol. The smallest standard deviation and coefficient of variation were determined at a temperature of 55 ℃, this temperature we used for further studies (Fig. 2). To study the sensitivity limit by real-time PCR, the sample was diluted to a in an amount of 5×10^5 CFU/cm³, which corresponds to the lowest dilution at which the reaction product was formed in conventional PCR. The research results are shown in Table 4.

The data of Table 6 indicate that the confidence interval of the analytical sensitivity of the method at in an amount of 1×10^5 CFU/cm³ and 1×10^4 CFU/cm³ was 100%. However, in an amount of 1×10^3 CFU/cm³, 1×10^2 CFU/cm³ and 1×10^1, the confidence interval of the analytical sensitivity of the method was 40%, 30%, and 10% respectively, and there was no detection result in an amount of less than 10 CFU/cm³.
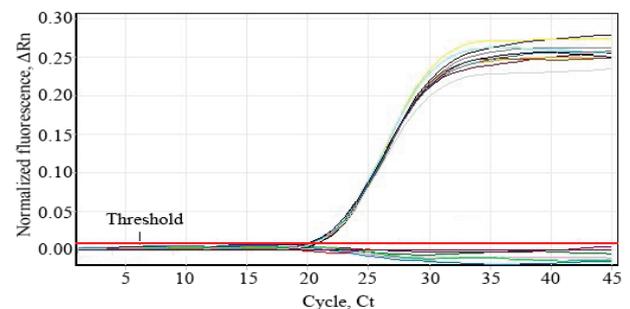
Fig. 2. Results of *S. suis* DNA amplification of studied samples to determine the convergence of the results obtained using the FAM/Green channel: ΔRn – relative units of fluorescence which are used to build a graph; Ct – cycle threshold

Table 3
Results of optimization annealing temperature gradient (Ta) real-time PCR

Sample		Annealing temperature gradient (Ta) which was used				
		50 ℃	53 ℃	55 ℃	57 ℃	60 ℃
Detected *Ct* in 10 repeats	*S. suis* NCTC 1023	17.61	20.79	21.03	21.79	23.17
	S. suis serotypes 1	17.63	20.83	21.19	21.65	23.36
	S. suis serotypes 2	18.32	20.36	20.47	20.86	21.56
	S. suis serotypes 2	18.85	20.23	20.71	21.45	22.36
	S. suis serotypes 1/2	21.23	20.35	20.74	21.39	22.44
	S. suis serotypes 1/2	21.59	20.69	20.84	21.96	22.69
	S. suis serotypes 5	25.17	21.36	20.37	21.16	21.39
	S. suis serotypes 7	25.23	21.14	20.42	21.75	22.14
	S. suis serotypes 10	28.36	21.63	20.65	21.15	22.73
	S. suis serotypes 14	29.51	21.48	20.63	21.63	22.15
	Mean Ct ± SD	22.35 ± 4.46	20.89 ± 0.50	20.67 ± 0.26	21.48 ± 0.34	22.40 ± 0.63

Note: "Ct" – cycle threshold.

Table 4

Detection limit for *S. suis* using real-time PCR

	Quantity of CFU in sample	1×10^5	1×10^4	1×10^3	1×10^2	1×10^1
	S. suis NCTC 1023	24.75	28.49	35.73	36.18	37.21
	S. suis serotypes 1	24.63	29.66	34.68	0	0
	S. suis serotypes 2	25.13	28.54	34.78	37.22	0
	S. suis serotypes 2	24.75	29.03	0	0	0
Detected *Ct* in 10 repeats	*S. suis* serotypes 1/2	24.47	29.22	0	0	0
	S. suis serotypes 1/2	25.38	28.76	35.27	36.15	0
	S. suis serotypes 5	25.84	28.67	0	0	0
	S. suis serotypes 7	24.70	29.68	0	0	0
	S. suis serotypes 10	25.24	28.62	0	0	0
	S. suis serotypes 14	24.85	28.64	0	0	0
Mean *Ct* ± SD		24.97 ± 0.42	28.93 ± 0.45	–	–	–
Positive/ negative results		10/0	10/0	4/6	3/7	1/10
Analytical sensitivity, %		100	100	40	30	10

Note: "–" – cannot be calculated due to the fact that the reaction did not occur in all samples.

Table 5

Evaluation of the convergence of the results
of *S. suis* DNA detection by real-time PCR

Number repeats of test	Ct, FAM
S. suis NCTC 1023	21.03
S. suis serotypes 1	21.19
S. suis serotypes 2	20.47
S. suis serotypes 2	20.71
S. suis serotypes 1/2	20.74
S. suis serotypes 1/2	20.84
S. suis serotypes 5	20.37
S. suis serotypes 7	20.42
S. suis serotypes 10	20.65
S. suis serotypes 14	20.63
Mean Ct ± SD	20.71 ± 0.26

Note: "FAM" – the name a channel for detection of the signal.

So the limit of detection of the test is 1×10^4 CFU/cm^3. The convergence of the research results was calculated by several repeats of real-time PCR test under the same conditions with the determination of the variation coefficient (CV %) estimated by Ct (Fig. 1, Table. 5).

According to the data given in Table 3, the minimum value of Ct was 20.37 and the maximum was 21.19, and the mean ± SD was 20.70 ± 0.26.

Our study showed that morphological and cultural microbiological diagnostic methods allow us to determine *S. suis* at the genus level. Determination of biochemical properties using the API 20 STREP test kit can identify only 1 and 2 serotypes of *S. suis*. Conventional PCR and real-time PCR have 100% specificity and can be used to identify *S. suis* of different serotypes. The real-time PCR method is 2–4 times more sensitive than conventional PCR depending on the serotype studied, and can be used to accurately identify streptococcal DNA in a bacterial suspension dilution up to 10,000 CFU/cm^3 (Table 6).

Table 6

Comparison of sensitivity limits and specificity of methods used in the study

Samples	Sample	Sensitivity limits of conventional PCR, CFU/cm^3	Sensitivity limits of real-time PCR, CFU/cm^3	Difference in sensitivity limits of methods, ~ CFU/cm^3	The level of differentiation by microbiological methods
S. suis NCTC 1023	positive control	1×10^5	1×10^1	10000	serotype level
S. suis serotypes 1	positive sample	1×10^6	1×10^3	1000	serotype level
S. suis serotypes 2	positive sample	1×10^5	1×10^2	1000	serotype level
S. suis serotypes 2	positive sample	1×10^6	1×10^4	100	serotype level
S. suis serotypes 1/2	positive sample	1×10^7	1×10^4	1000	genus level
S. suis serotypes 1/2	positive sample	1×10^5	1×10^2	1000	genus level
S. suis serotypes 5	positive sample	1×10^6	1×10^4	100	genus level
S. suis serotypes 7	positive sample	1×10^6	1×10^4	100	genus level
S. suis serotypes 10	positive sample	1×10^7	1×10^4	1000	genus level
S. suis serotypes 14	positive sample	1×10^6	1×10^4	100	genus level
Staphylococcus aureus	negative control	absent	absent	absent	not posible
Micrococcus luteus	negative control	absent	absent	absent	not posible
Enterococcus faecalis	negative control	absent	absent	absent	species level
Streptococcus agalactiae	negative control	absent	absent	absent	species level

Discussion

The Ukraine veterinary service recommends routine testing of biological material from pigs to detect presence of *S. suis*. However, in Ukraine there are no official policies for the surveillance for *S. suis* in newborn pigs. With the standard bacteriological methods, including fermentative properties detection method, the percentage of positive test samples was low in comparison to the PCR assays. The affiliation of the pathogen to the genus *Streptococcus* can be detected by morphological and cultural properties. The pathogenic potential of the microorganism can be determined by the results of cultivation on a media containing erythrocytes. Pathogenic microorganisms cause hemolysis of erythrocytes in a nutrient media. The study of enzymatic properties is complicated by their variability among different serotypes. Commercial API20 STREP test kit is specially designed for differentiation of streptococci. According to the instructions, the test can identify only 1 and 2 types of *S. suis*, which we confirmed in our study. The matrix of description is absent for other serotypes. Given the wide genetic diversity of all types of streptococci, this can lead to false-negative research results. We have not found any research

about developing alternative enzyme tests. In our previous study, we found 100% specificity of the conventional PCR method, and the presence of *S. suis* DNA in the sample was confirmed by the multiplex PCR of the specific nucleotide sequences to individual serotypes of the pathogen. Our results were agreed with the results of the study (Groves et al., 2015; Tarasov et al., 2021).

In our study, we confirmed previously obtained results and additional studies of the analytical sensitivity of the method. The last in an amount of bacterial culture for which the 100% analytical sensitivity of the method was 1×10^7 (CFU/cm^3), at 1×10^5 the analytical sensitivity was 30%. van Samkar et al. (2015) also evaluated the use of conventional PCR as a method of detecting *S. suis* with high analytical sensitivity (100%). In a study of real-time PCR, we also found high values of 100% specificity. This method has a significantly higher analytical sensitivity, allows the pathogen to be determined with 100% analytical sensitivity in an amount of 1×10^4 (CFU/cm^3). The DNA of the museum strain we were able to determine in an amount of 1×10^1, which corresponds to the minimum in the amount of cells used in our study. The specificity of the PCR test has to be compared with the results of the microbiological culture identifica-

tion. A useful approach is provided by a confirmary test like the one presented by bioMerieux, Api Strep. However, the use of a special blood containing broth is necessary for the detection of hemolytic properties. This means that the results considered as false-positives in the PCR techniques are in fact true positives, because of the greater analytical sensitivity of the molecular method (Srinivasan et al., 2016; Yang et al., 2016) of properties' detection. In the results published by Feuerschuette et al. (2012), the samples with discrepant results, negative by the culture and positive by real-time PCR, were submitted to conventional PCR, which provided mainly positive results. The results obtained with real-time PCR were thus considered true positives, while the negative results with culture were considered false negative. Thus, the high specificity of the PCR techniques may result in more precise detection of *Streptococcus* causative agents in samples where the culture method fails.

It is therefore relevant that the chosen methodology for routine screening programs has a high analytical sensitivity, such as the one verified in the real-time PCR assay in this study, which was capable of detecting amounts as low as 10^2 copies/sample of *S. suis*. The limit of detection indicated in our assay is superior to the one described by Dekker et al. (2016), who performed a real-time PCR assay with the same target gene. They evaluate a minimum limit of detection of 18 copies/µL. The high analytical sensitivity limit of the test is an important parameter when screening is the focus, and obtaining false negative results carries the risk of infection among the infected pig population. PCR methods are evaluated as being suitable for screening procedures due to their high analytical sensitivity limit and their being less time consuming compared to traditional microbiological methods (Chen et al., 2013; Ishida et al., 2014). The PCR approach is important because it indicates the possibility of correct detection of negative clinical samples. The real-time PCR method had better performance in obtaining positive results compared to conventional PCR (Arai et al., 2018).

These results may be due to the fact that conventional PCR is a qualitative reaction. Identification of results depends on the researcher. The researcher receives real-time PCR results in digital form. This reduces the window of possibility of interpretation of the method, and allows one to use statistical calculations at the stage of protocol optimization. The real-time PCR reaction takes less time than conventional PCR because amplicon application and detection take place simultaneously. The measurement results are immediately digitized, the data can be subjected to statistical processing. This reduces the time spent on reacting and analyzing the results. The limitation of molecular genetic methods is that they provide information only about the presence of genetic material of the infectious agent.

Real-time PCR allows one to estimate only the initial amount of the pathogen. However, the presence of the pathogen does not indicate its pathogenic potential or participation in the local infectious process. Microbiological methods make it possible to assess the pathogenetic potential of the pathogen, but are not effective for its accurate identification. Also, the selection of pure culture is necessary to establish the profile of antibacterial resistance. We propose to combine these two groups of research methods. Morphological and cultural properties of the pathogen will indicate its belonging to the family of streptococci and pathogenetic potential. The isolated pure culture will be identified as pathogenic or non-pathogenic streptococcus. At the stage of dilution of the bacterial suspension to 1×10^8, for seeding to determine antibacterial resistance, the material needs to be taken for DNA isolation. Real-time PCR will accurately identify the pathogen as *S. suis*. DNA is easy to store, and can then be used to serotype the pathogen by conventional PCR or other molecular-genetic methods.

Given the sensitivity limit of real-time PCR, we suggest the possibility of identifying the pathogen in the synovial fluid of the joints or cerebrospinal fluid from native material. Detection of the pathogen in these fluids will indicate its pathogenic potential, because normally these materials must be sterile. The high specificity of PCR methods can lead to more accurate detection of the streptococcus causative agent in samples where the culture method fails. The limitation of our study is that it was not possible to estimate the DNA concentration after isolation from a sample of bacterial cell suspension, so we indirectly estimated the DNA concentration in the sample by the number of bacterial cells (CFU/cm^3) in the primary sample. It was not possible to measure the number of bacterial cells in the suspension by the instrumental method through optical density (e.g. turbidimetry), so we used the available method according to the McFarland standard.

We did not have the opportunity to repeat the real-time PCR study using 30 cycles, so we focused on the analysis of results instead of real-time PCR results with 35 cycles. Overcoming these limits would clarify our results but would not affect the conclusions that are quite achievable in our research.

Conclusions

As a result of the studies, it was found that the use of the real-time PCR method makes it possible to detect *S. suis* in an amount of 1×10^4 copies of the genome in the sample. Among other studied methods (microbiological and conventional PCR), the real-time PCR method proved to be the most sensitive. A microbiological method is available for the diagnosis of *S. suis*, which is considered the standard method of diagnosis. Also available are molecular diagnostic methods for conventional PCR and real-time PCR, which we have used and evaluated in this article. All three methods can be used to diagnose *S. suis* in pathological material for a definitive diagnosis. We optimized the research protocol of real-time PCR as the most promising research method. But as a result, it was determined that the lowest amount for successful diagnosis was 1×10^4. A protocol with optimized temperatures of 53 °C (SD 0.50%, CV 2.39%), 55 °C (SD 0.26%, CV 1.26%) and 57 °C (SD 0.34%, CV 1.59%) was proposed. The lowest standard deviation and coefficient of variation were determined using a temperature of 55 °C, this temperature we used for further research. The validation parameters of the qualitative method for determining *S. suis* DNA by real-time PCR which meet international requirements (ISO 17025) have been optimized, which guarantees accurate and reliable results.

Based on the results of the study, we recommended that laboratories for monitoring and controlling the spread of porcine streptococcosis use real-time PCR to identify *S. suis*.

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