A polymerase chain reaction (PCR) assay specific for *Streptococcus suis* based on the gene encoding the glutamate dehydrogenase

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Abstract

Polymerase chain reaction (PCR) primers that flank a 688-bp segment within the glutamate dehydrogenase gene (*gdh*) of *Streptococcus suis* type 2 could amplify efficiently the DNA of all 306 (100%) clinical *S. suis* isolates tested (pigs, *n* = 305; human, *n* = 1) encompassing all serotypes obtained from diverse organs, and geographic origins. When DNA from other bacteria were used as templates for amplification, no product was detected indicating specificity of the primers. Multiplex PCR was developed using the *gdh* gene primer pair and primers that targeted the gene encoding *S. suis* capsular biosynthesis (*cps*). This strategy enabled the detection of strains belonging to serotypes 1/2, 1, 2, 7, and 9, respectively. Using the multiplex-PCR technique, 12 out of 14 (86%) isolates that were previously identified as nontypable *S. suis* (based on biochemical reactions and serology) gave positive PCR results of which four were positive for serotype 7, three for serotype 2, and five for *S. suis* strains that belong to other serotypes. Retest results of all 14 isolates by several veterinary laboratories were identical with PCR and confirmed that the two non-PCR reactive isolates belonged to strains of other streptococcal species. These results indicated that PCR improved species determination and can thus be used as a reliable species-specific molecular diagnostic reagent for the accurate identification of *S. suis* isolates and a serotype-specific method for the detection of strains of serotypes 1/2, 1, 2, 7, and 9, respectively. The PCR method therefore has potential clinical and epidemiological applications.

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Keywords: *Streptococcus suis*; Glutamate dehydrogenase; Polymerase chain reaction; Capsular biosynthesis

1. Introduction

*Streptococcus suis* is an important pathogen of swine responsible for a wide range of diseases, such as meningitis, arthritis, septicaemia, endocarditis, encephalitis, abortions, polyserositis, and bronchopneumonia. Outbreaks have been recognized worldwide in countries where hog production is well developed. Subclinical carrier animals are the most important source from which the bacteria are transmitted to susceptible young pigs. *S. suis* has also been isolated from other animal species and from humans [12]. All reported human cases have been associated with biochemical reactions and serology gave positive PCR results of which four were positive for serotype 7, three for serotype 2, and five for *S. suis* strains that belong to other serotypes. Retest results of all 14 isolates by several veterinary laboratories were identical with PCR and confirmed that the two non-PCR reactive isolates belonged to strains of other streptococcal species. These results indicated that PCR improved species determination and can thus be used as a reliable species-specific molecular diagnostic reagent for the accurate identification of *S. suis* isolates and a serotype-specific method for the detection of strains of serotypes 1/2, 1, 2, 7, and 9, respectively. The PCR method therefore has potential clinical and epidemiological applications.

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1. Introduction

*Streptococcus suis* is an important pathogen of swine responsible for a wide range of diseases, such as meningitis, arthritis, septicaemia, endocarditis, encephalitis, abortions, polyserositis, and bronchopneumonia. Outbreaks have been recognized worldwide in countries where hog production is well developed. Subclinical carrier animals are the most important source from which the bacteria are transmitted to susceptible young pigs. *S. suis* has also been isolated from other animal species and from humans [12]. All reported human cases have been associated with slaughterhouse workers handling infected pork, and deaths have been reported among those workers [12].

Strains of *S. suis* are divided into serotypes according to polysaccharide capsular antigens. Thirty-five capsular serotypes (types 1/2 and 1 through 34) have been identified of which type 2 is considered to be the major cause of disease and is also the most frequently isolated serotype. Strains belonging to other serotypes such as types 1/2, 1, 7, 9, and 14 can also cause disease [12].

Attempts to control *S. suis* related diseases are still hampered by the lack of knowledge about the epidemiology of the disease and the lack of sensitive diagnostics and effective vaccines. Because this organism can cause severe and costly health problems in pigs and because humans working with infected animals are at risk, the development of a reliable, rapid, sensitive, and specific diagnostic assay is needed for its detection and control.

The detection of *S. suis* is dependent on standard culture methods and serotyping [12]. These methods are labor intensive and results can be ambiguous or inconclusive. Thus, detection and control of *S. suis* infection depends increasingly on the availability of rapid and precise diagnostic tests.
A polymerase chain reaction (PCR) technique has been evaluated previously for *S. suis* [7]. Results showed that the targeted DNA regions were not conserved across capsular types or pathogenic strains. Additionally, the presence or lack of the targeted genes in several isolates was influenced by geographical location [7]. Recently, Smith et al. [10,11] showed using a monoplex-PCR format, that primers derived from the gene encoding *S. suis* capsular biosynthesis (cps) were specific for *S. suis* serotypes 1/2, 1, 2, 7, 9, and 14, respectively, which are the serotypes of greatest concern. However, this PCR approach requires independent PCR reaction mixtures and thermocycler parameters for each primer pair, which is time consuming and prone to errors. Thus, a single PCR assay that permits detection of multiple serotypes would be the preferred method.

Okwumabua et al. [8] recently cloned the gene encoding the glutamate dehydrogenase (GDH) of *S. suis* type 2. They showed that, like other GDHs, the *S. suis* gdh gene appeared to be conserved [8]. GDH has been successfully used in the diagnosis of certain other bacterial infections [4] and it reportedly is highly conserved and exhibits an extremely low rate of point mutation relative to other genes [1]. On the basis of these observations, and in view of the limitations of the available *S. suis* diagnostic methods, we hypothesized that the *S. suis* gdh gene will be a good target for the development of a diagnostic reagent for the identification of *S. suis* from clinical samples.

In this study, we report the development of a gdh-based PCR assay for the rapid detection of *S. suis* that is specific and sensitive, and can detect *S. suis* isolates regardless of serotype, or geographic origin. By employing previously published sets of serotype-specific primers [10,11] in a multiplex-PCR format, strains belonging to the disease causing *S. suis* serotypes (serotypes 1/2 and 2, 1, 7, and 9) can be distinguished from each other and from other serotypes. Thus, this PCR method is attractive for use in clinical laboratories and for epidemiological purposes.

### 2. Materials and methods

#### 2.1. Bacterial strains

Three hundred and five clinical isolates of *S. suis* strains used in this study encompassing all serotypes recovered from different organs of infected pigs were obtained from varied geographical locations including Canada, Denmark, The Netherlands, and the United States of America. The single human *S. suis* serotype 2 isolate (ATCC 700796) recovered from the brain was obtained from the American Type Culture Collection (Manassa, VA, USA). Fourteen strains recovered from pigs and designated as non-typable *S. suis* based on their similar biochemical profile to that of *S. suis* [3,13] and lack of reactivity with the 35 different sera used in the serotyping of *S. suis* were obtained from Dr. Mike Titus (Biovet Inc., St. Anthony, MN, USA). The non-*S. suis* isolates, consisting of several Gram-positive bacteria, including streptococcal species and Gram-negative bacteria, were obtained from different regions of the United States. Identity of all strains was confirmed by standard procedures.

#### 2.2. DNA extraction

Lysis by a boiling method was used. Briefly, a single colony of the test bacterial isolate grown on sheep blood agar plate was suspended in 100 μl of HPLC grade water and heated at 100°C for 20 min in a heat block ( Fisher Scientific) to lyse cells. Following centrifugation for 2 min at 13,000 × g in a microcentrifuge, the cell-free supernatant containing DNA was transferred to a fresh tube and used for PCR.

#### 2.3. Primers

Oligonucleotide primers were designed by using the gdh nucleotide sequence data [8]. Based on multiple sequence alignments with gdh genes from various sources at the GenBank nucleotide database, regions of the gdh gene with the least similarity to gdh from other species were chosen, and PCR primers designated JP4 (5'-GCAGCG-TATTTCTGTCAAACG-3') and JP5 (5'-CATGGAACA-GATAAAGATGG-3') were derived from these regions with the aid of the MacVector software package (Oxford Molecular Group, Inc., Campbell, CA, USA). The primer pair will result in the amplification of a 688-bp fragment. The serotype-specific primers for *S. suis* serotype 1 (cps1I-1 and cps1I-2), serotype 7 (cps7-1 and cps7-2), and serotype 9 (cps9H-1 and cps9H-2) have been described elsewhere [10,11]. For serotype-specific primers for *S. suis* types 1/2 and 2 we designed a forward primer designated cps2J-2 (5’-TGATAGTGATTTGTCGGGAGGG-3’) from the cps nucleotide sequence (GenBank accession number AF118389) and used in pair with a previously published cps2J-F primer [11]. This will result in the amplification of a 575-bp fragment that differs in size from the product of JP4 and JP5 primer pair. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA).

#### 2.4. PCR amplification

Amplification reactions were performed in a total volume of 50 μl containing 10 mM Tris–HCL (pH 8.3); 1.5 mM MgCl₂; 50 mM KCl; 0.001% gelatin; 200 μM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP); 1 μM of each primer; 2.5 U of Taq polymerase (Perkin Elmer Corp., Norwalk, CT, USA) and 5 μl of boiled cell lysate for the monoplex PCR. The PCR assay was carried out in a Perkin Elmer 2400 thermocycler, comprising 5 min of pre-incubation at 94°C, followed by
35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Final extension was performed for 7 min at 72°C. DNA from S. suis type 2 strain 1933 [8] was used as the positive control template. The negative control was a reaction mixture containing all reagents but no DNA template. For multiplex PCR, all conditions were identical to the monoplex PCR except that 0.5 μM of each primer was used. The PCR products were visualized by electrophoresis on a 1% agarose gel following standard procedures. All PCR negative samples were spiked with the positive control DNA.

Table 1
List of bacteria used in this study

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<tr>
<th>S. suis serotypes</th>
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Other streptococcal species (n = 9)
- Streptococcus dysgalactiae
- Streptococcus zooepidemicus
- Streptococcus uberis
- Streptococcus equisimilis
- Streptococcus pyogenes
- Streptococcus pneumoniae
- Streptococcus equi
- Streptococcus bovis
- Streptococcus suis

Table 1 (Continued).

Other bacteria (n = 18)
- Acanobacterium pyogenes
- Aeromonas hydrophila
- Escherichia coli
- Haemophilus somnus
- Klebsiella pneumoniae
- Pasteurella multocida
- Mannheimia hemolytica
- Pseudomonas aeruginosa
- Salmonella species
- Campylobacter jejuni
- Campylobacter fetus
- Actinomyces pyogenes
- Bordetella bronchiseptica
- Campylobacter coli
- Mycoplasma hyosynoviae
- Mycoplasma hyopneumonia
- Haemophilus parasuis
- Enterobacter species

Other Gram-positive bacteria (n = 9)
- Bacillus subtilis
- Bacillus licheniformis
- Clostridium perfringens
- Listeria monocytogenes
- Staphylococcus intermedius
- Staphylococcus aureus
- Enterococcus faecalis
- Micrococcus species
- Mycobacterium paratuberculosis

2.5. Specificity

The specificity of the assay was verified by using DNA from a battery of isolates representing a wide variety of Gram-positive and Gram-negative bacterial species as the template for PCR reactions (Table 1). A total of 306 clinical isolates of S. suis were also tested to further validate the ubiquity of the JP4 and JP5 primer pair (i.e. the ability to detect all S. suis strains irrespective of serotype) in the S. suis-specific gdh-based PCR assay.

3. Results

3.1. DNA extraction

Because of the need for a quick turnaround time and simplicity, we utilized lysis by boiling as a means for template preparation. The method yielded sufficient amounts and quality of DNA to yield amplified products from S. suis, thus eliminating laborious and time-consuming extraction procedures (Fig. 1).

3.2. Specificity of the gdh gene primers

The PCR primers (JP4 and JP5) designed from the
S. suis gdh gene, amplified the DNA from all 306 S. suis strains tested and yielded the expected 688-bp product (Fig. 1). No false negatives were encountered. To further test the diagnostic value of the S. suis gdh-based PCR assay as it relates to specificity, DNA from non-S. suis bacteria (streptococcal species, n = 9; other Gram-positive species, n = 9; and other bacteria including Gram-negative species, n = 18) were used as the templates for PCR (Table 1). No amplification product was detected, indicating specificity of the primers (Fig. 2). When samples from the PCR negative non-S. suis isolates were spiked with S. suis DNA, amplification product was detected only in the spiked samples, indicating the absence of inhibitor (Fig. 2).

Because serotype 2 is the most frequently associated with disease, and is the most frequently isolated S. suis serotype, and because serotypes 1/2, 1, 7 and 9 have also been associated with disease, we tested whether the PCR assay could be formulated to detect these serotypes using primers from the gdh gene and the cps locus, respectively, in a multiplex-PCR format. As expected, the gdh gene primers (JP4 and JP5) amplified a 688-bp fragment from all strains tested. Cps2J-F and Cps2J-2 primers amplified a 575-bp fragment from strains of both serotypes 1/2 and 2, respectively (Fig. 2). Our efforts with several other combinations of primers to distinguish serotype 1/2 from type 2 were not successful. Consistent with a previously published report [10,11] Cps1I-1 and Cps1I-2 primers specific for serotype 1, Cps9H-1 and Cps9H-2 primers specific for type 9, and Cps7-1 and Cps7-2 primers specific for type 7 yielded the expected fragment sizes of 440-, 390-, and 250-bp products, respectively (Fig. 3). Similar to the results obtained using serotype 2-specific primers and consistent with previous observations [11] primers specific for serotype 1 also detected strains of serotype 14.

By the multiplex-PCR technique, 12 out of 14 S. suis isolates that were originally identified and designated as non-typable S. suis strains gave positive PCR results, in agreement with biochemical analysis. Four of these isolates were identified as serotype 7, three as serotype 2 and five as S. suis strains that belong to serotypes other than 1/2, 1, 2, 7 or 9 (data not shown). Upon retest of the 14 isolates by serotyping, results agreed also with that of PCR and confirmed that the two non-PCR reactive isolates belonged to other streptococcal species. These results indicated that the two isolates had been incorrectly identified by the biochemical method and that PCR was more definitive and revealed the inherent problem associated with the interpretation of biochemical and serotyping results.

4. Discussion

S. suis infection is one of the major causes of sudden death of pigs resulting in substantial economic loss to the
Swine industry. *S. suis* infections are also increasingly becoming a human health concern by virtue of its zoonotic capabilities [12]. The diagnosis of *S. suis* infection is generally based on clinical criteria and the results of conventional biochemical and serological tests. These methods are time consuming, laborious, and do not unequivocally distinguish *S. suis* from other related organisms. The development of a sensitive DNA-based assay for the identification of *S. suis* isolated from clinical specimens may improve the rapidity and accuracy of the diagnosis.

One of the difficulties associated with the development of a diagnostic assay, and a heterologous vaccine for *S. suis* is that strains of this organism exhibit extensive genetic heterogeneity within and between serotypes [2,6]. Thus, identification of an antigenic factor or DNA region conserved across capsular types or pathogenic strains irrespective of geographic origin may help to obviate this problem.

In the present study, we have developed a *S. suis*-specific PCR-based assay that targeted the *gdh* gene. GDHs reportedly are highly conserved and exhibit an extremely low rate of point mutations relative to many other genes [1]. In addition, GDH has been successfully used in the diagnosis of the infections caused by *Clostridium difficile* [4]. Analogous to GDH of *C. difficile*, the *S. suis gdh* gene is conserved across *S. suis* capsular types irrespective of geographic origin. Thus, primers derived from within the gene are species-specific and are of diagnostic importance.

With respect to DNA extraction, boiling of the samples has been shown to be a simpler and economical method for releasing DNA from bacteria [9]. It also appears to be the most attractive and efficient method for the extraction of *S. suis* DNA due to its rapidity and simplicity, and it compared unequivocally with other DNA extraction methods [5,9] that we evaluated (data not shown).

There are 35 capsular types of *S. suis* described to date. Only the strains that belong to serotypes 1/2, 1, 2, 7, 9, and 14 have been reported to be associated with disease [12]. Recently, Smith et al. [10,11] reportedly developed a monoplex-PCR assay that targeted the *S. suis* capsular biosynthesis (*cps*) locus for the specific detection of strains of these serotypes. We employed most of their set of published primers in a multiplex-PCR format, enabling us to distinguish these strains from those of other serotypes. This feature is especially advantageous because an assay that correctly identifies *S. suis* isolates at the species level and differentiates *S. suis* strains that belong to the disease-causing serotypes is highly desirable for effective implementation of control measures. Because the primers for serotype 1 detect strains of serotype 14, and those of serotype 2 detect also strains of serotype 1/2 [11], serotyping may be required in these situations to aid distinction.

Regarding the 14 non-typable isolates, the fact that 12 were positive by PCR at the species level indicated that the PCR assay was in agreement with biochemical test on those isolates. Because seven of the isolates were successfully serotyped upon retest with fresh sera and also identified by PCR to the serotype level, the initial failure to serotype them could be attributed to the use of poor quality sera or undetermined technical mistakes. The two non-typable and PCR negative isolates that later were con-

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**Fig. 3. Ethidium bromide stained agarose gel after electrophoresis of multiplex-PCR products with primers described in Section 2.** Lane M: ΦX174 DNA/HaeIII molecular mass markers; lanes 1, 2: *S. suis* serotype 1/2; lanes 3, 4: *S. suis* serotype 2; lanes 5, 6: *S. suis* serotype 1; lanes 7, 8: *S. suis* serotype 7; lane 9: *S. suis* serotype 9; lane 10: *S. suis* serotype 3; lane 11: negative control (no template). The expected migration positions of the amplicons are indicated.
firmed to belong to other streptococcal species indicated that biochemical test cannot be relied upon entirely for accurate identification of S. suis due to mistakes in interpreting results and gave credence to the PCR assay.

In summary, the results presented here indicate that a multiplex-PCR technique can be used successfully for the rapid detection of S. suis. The technique is definitive, and results are easy to interpret compared with other methods available for identification of S. suis. Thus, it is a valuable addition to the methods used to diagnose S. suis infections. Compared to other molecular techniques, PCR is more versatile and should be easily maintained in laboratories in regions where S. suis is problematic.

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References