Molecular typing of Streptococcus suis *from pigs in Cuba*

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RESEARCH

ABSTRACT

Streptococcus suis is a bacterium commonly carried by pigs in the respiratory tract; thus the infections caused by virulent strains are considered a problem in the swine industry. A successful approach for the identification of virulent strains is the differentiation of capsular serotypes using specific antisera or the corresponding *cps* types by genotypic assessment, with the subsequent detection of virulence associated factors, namely the extracellular factor, the muramidase-released protein and the hemolysin suilysin. Data regarding serological and molecular identification of *S. suis* from pigs in Cuba are not available. This study was aimed at identifying the capsular types *cps*2, 7 and 9, as well as three genes related to virulence using PCR assays. According to the results, 31 isolates were evaluated and classified as *cps*2 (n = 21) or *cps*9 (n = 4), while six isolates not were typable. Considering the presence in these isolates of the genes *mrp*, epf and *sly*, six different genotypes were differentiated among the *cps*2 or csp9 strains and there were three non-typable isolates for the genes used in this study. The *cps*2 isolates were recovered from pigs between 6-12 and 14-17 weeks with pneumonia and systemic infection respectively, whereas the *cps*9 isolates were exclusively associated with pneumonia.

Keywords: *Streptococcus suis*, virulence markers, genotypes

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RESUMEN

Tipificación molecular de *Streptococcus suis* aislado de cerdos en Cuba. *Streptococcus suis* es una bacteria común en el tracto respiratorio de los cerdos; sin embargo las infecciones causadas por cepas virulentas se consideran un problema en la industria porcina. Estas cepas se pueden identificar por la diferenciación de los serotipos capsulares con antisueros específicos o por sus correspondientes tipos *cps*, mediante ensayos genotípicos. También se pueden reconocer mediante la detección de factores asociados con la virulencia, como el factor extracelular, la proteína liberada por muramidasa y la hemolisina suilisina. Antes de esta investigación no había datos sobre la identificación serológica o molecular de los tipos capsulares de *S. suis* procedentes de cerdos en Cuba. El objetivo de esta investigación fue la detección de los tipos capsulares *cps*2, *cps*7 y *cps*9, y de los tres genes asociados con la virulencia, mediante ensayos de reacción en cadena de la polimerasa. De 31 aislamientos, 21 se clasificaron con el genotipo capsular *cps*2, y 4 con el *cps*9. El genotipo capsular *cps*7 no se detectó y 6 aislados no se correspondieron con ninguno de los serotipos analizados. Considerando la presencia de los genes *sly*, epf y *mrp*, se identificaron seis genotipos en los aislados *cps*2 y *cps*9, y 3 en los aislados cuyo genotipo *cps* no se identificó. Los aislados *cps*2 se extrajeron de cerdos de 6 a 12 con neumonía y de 14 a 17 semanas con infección sistémica; mientras que los *cps*9 estaban asociados exclusivamente con neumonía.

Palabras clave: *Streptococcus suis*, marcadores de virulencia, genotipos

Introduction

Streptococcus suis has emerged as an important swine pathogen in most countries with an intensive swine production [1]. *S. suis* infections can be the cause of meningitis, polyarthritis, polyserositis, endocarditis, septicaemia, pneumonia and sudden death, leading to significant economic losses in pig farms [2-6]. This bacterium is also a zoonotic agent responsible for meningitis and arthritis in humans in close contact with infected pigs [6-8].

S. suis is a heterogeneous species that can be divided into 33 serotypes with respect to the capsule polysaccharides. Worldwide, capsular serotype 2 (*cps*2) is the most prevalent one among invasive porcine and human isolates [9, 10]. However, the importance of particular serotypes can vary geographically. Serotypes *csp9* and *csp7* are particularly reported common in Europe [4]. Major parts of the *cps* loci that are responsible for the biosynthesis of the capsule polysaccharides of the greatest concern serotypes 1 (and 14), 2 (and 1/2), 7 and 9, have been sequenced [11-14]. PCR tests based on sequences of type-specific capsular genes have been developed, for other serotypes diagnostic methods (although unreliable) are available [11-14].

Virulence can vary substantially both within and among serotypes, and not all isolates of the same serotype cause the same disease [4]. Although little is known about potential virulence factors, such as the hemolysin suilysin (SLY; encoded by the *sly* gene), the muramidase-released protein (MRP; *mrp*), and the extracellular factor (EF, *epf*) are virulence markers that have been used in elaborated genotypic and phenotypic schemes to try to predict the virulence of a given *S. suis* strain [15-17]. It is now included in routine diagnosis of *S. suis* in several laboratories [4].

In Cuba, the pig production has increased since 1997, and an intensive technology is being practiced

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in a considerable number of farms (30 % of the swine population) [18]. However, data regarding *S. suis* isolates typing from pigs in Cuba are not available. In this work, the molecular identification of the capsule polysaccharide genes (*cps*) and the genotypic assessment of virulence markers in Cuban *S. suis* isolates were carried out for the first time.

Materials and methods

Samples and classification

A total of 31 isolates of *S. suis* from the collection of the Bacteriology Department of the National Center of Animal and Plant Health (Censa, Cuba) were included in this study. Most of these isolates were collected from diseased or dead animals during diagnostic procedures from farrow-to-finish farms in the Western region of Cuba from 2004 to 2011.

According to their source of isolation and clinical symptoms, the isolates were distributed in three groups as follows: i) Invasive group (n = 3), three isolates from abscess of pigs 10-17 weeks of age with arthritis and one isolate from aborted foetus; ii) pneumonia group (n = 21), isolates from the lung of animals between 10-12 weeks of age; and iii) carrier group (n = 6), comprising two isolates of tonsillar biopsies from healthy animals, one isolate from the vaginal secretion and three isolates of semen ejaculates from mature boars of a pig breeding farm.

PCR

The isolates were identified by conventional biochemical procedures following the methods reported by Berthelot-Herault *et al.* [19]. The isolates were cultured in Todd Hewitt medium (5 mL) at 37 °C for 24 h and the DNA was extracted as described previously [20]. The *S. suis*-like strains were identified by amplification of a 294-bp-long 16S rDNA gene fragment by PCR using *S. suis* species-specific primers [13]. The capsular genotype (*cps*2) was determined by PCR as previously described by Smith *et al*. [12]. Capsular genotypes *cps*7 and *csp*9, and the *mrp* (188 bp) gene were analyzed by monoplex PCR assays using the sequence of primers and conditions described previously by Silva *et al*. [14]. Other two conventional PCR assays were used to screen the presence of *sly* (1492 bp) [21] and *epf* (626 bp) genes [17]. Details of all oligonucleotide primers used and PCR conditions applied for the detection are listed in table 1. Each isolate was tested under the same conditions twice.

The reaction was carried out in a 25-µL PCR mixture containing 20 pmol of each primer, 200 µM of dNTPs, 1× PCR buffer, 3 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). The amplified DNA was visualized in 2 % agarose gels in TBE buffer (90 mM Tris, 90 mM borate, 2.5 mM EDTA; pH 8) for 1 h at a constant voltage of 125 V. Amplified products were stained with ethidium bromide (0.5 µg/µL) and detected by UV transillumination. The 50 bp ladder (Promega) was used as standard.

DNA sequencing

Partial sequences corresponding to *cps*2 and 16S rDNA genes were amplified by using chromosomal DNA obtained from sample 13 (designated as isolate SS13) collected in October 2010 from the lung of a pig with pneumonia. The sequences of both genes were determined by an automated sequence analyzer (CEQ™8800, Beckman Coulter, USA) using the same primers as for PCR amplification and Genome Lab™ DTCS- Quick start kit. Prior to the sequencing reaction, PCR products were purified with the Wizard PCR purification kit (Promega) according to the manufacturer's instructions.

Table 1. Oligonucleotide primer sequences and PCR conditions applied for the detection of virulence associated genes in *S. suis* strains used in this study

Gene	Primer sequence (5´-3´)	PCR product (bp)	PCR conditions (°C/s)		
			Denaturation	Annealing	Elongation
16S rDNA	cagtatttaccgcatggtagatat gtaagataccgtcaagtgaga	294	94/30	60/30	72/60
*cps*2	caaacgcaaggaattacggtatc gagtatctaaagaatgcctattg	652	95/60	56/120	72/120
*cps*7	aatgccctcgtggaatacag aatgccctcgtggaatacag	379	94/60	58/60	72/120
*cps*9	ggctacatataatggaagccc ccgaagtatctgggctactg	303	95/60	56/120	72/120
mrp	attgctccacaagaggatgg tgagctttacctgaagcggt	188	94/60	58/90	72/60
epf	gctacgacggcctcagaaatc tggatcaaccactggtgttac	626	94/60	55/60	72/120
sly	aagtcgacatgagaaaaagttcgcac aactgcaggattactctatcacctca	1492	94/60	56/60	72/60

Sequence analysis

Sequences of both fragments corresponding to *cps*2 and 16S rDNA genes were aligned and assembled using the Vector NTI® software (Invitrogen, USA) to obtain two sequences of 260 and 637 bp, which were deposited in GenBank under accession numbers JF266696 and JF266697, respectively. They were aligned and studied using the MEGA5 software [22] for phylogenetic inference by using the Neighbor-Joining method [23]. The evolutionary distances were computed using the Kimura 2-parameter method [24]. The analysis involved 22 nucleotide sequences. To obtain a confidence value for the aligned sequence dataset, a bootstrap analysis [25] of 500 replications was done.

Results and discussion

All isolates included in this study showed a 294 bp fragment corresponding to 16S rDNA under the conditions described by Marois *et al*. [13]. The four isolates originating from animals with invasive procedures and the 21 isolates from pig lungs with pneumonia were isolated in pure culture. *S. suis* is an invasive pathogen and a very successful colonizer of mucosal surfaces, in particular of the upper respiratory tract. Reports about the isolation of this bacterium from pigs suffering arthritis and pneumonia are frequent [4]; thus, a potential causative role of this organism in respiratory diseases is also suggested [26]. For instance, most clinical *S. suis* isolates in Canada were from piglet lungs [27].

In addition, *S. suis* was identified in samples of tonsil, semen and vaginal discharge in healthy animals (Table 2). Dee and Corey [28] reported that *S. suis* strains can maintain their viability in different porcine

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Table 2. Distribution of *S. suis* genotypes among different sources of isolation (n = 31)

Isolate	Genotype	Isolation site (n)					
		Vaginal discharge (1)	Lung (21)	Joint (3)	Aborted fetus (1)	Semen (3)	Tonsil (2)
cps2	*cps2*$^+$ *mrp*$^+$ *epf*$^+$ *sly*$^+$		1				
	cps2$^+$ *mrp*$^+$ *epf*$^-$ *sly*$^+$		2				
	cps2$^+$ *mrp*$^+$ *epf*$^-$ *sly*$^-$		2			1	
	cps2$^+$ *mrp*$^+$ *epf*$^+$ *sly*$^-$		1				
	cps2$^+$ *mrp*$^-$ *epf*$^-$ *sly*$^-$	1	8	3	1		1
cps9	*cps9*$^+$ *mrp*$^+$ *epf*$^-$ *sly*$^-$		4				
Non-type	*mrp*$^-$ *epf*$^-$ *sly*$^+$						1
(*cps2*$^-$ *cps7*$^-$ *cps9*$^-$)	*mrp*$^+$ *epf*$^-$ *sly*$^-$		1				
	mrp$^-$ *epf*$^-$ *sly*$^-$		2			2	

fluids, such as urine or semen, up to 10 days. When *S. suis* is isolated from the tonsils and the genital and alimentary tracts of carrier pigs, those animals are discarded for being a source of *S. suis* transmission in herds [29, 30].

The 31 isolates of *S. suis* tested in this study demonstrated a high genetic diversity, being grouped into nine different genotypes with the aid of the previously described PCR assay (Table 2). Only genotypes *cps*2 (68 %) and *cps*9 (13 %) were detected, and six isolates from tonsils and semen of asymptomatic animals were non-typable for the three capsular genotypes investigated. The *cps*9 genotype was identified exclusively in isolates from lungs, while genotype *cps*2 was found in different isolates regardless of their origin (*i.e.*, lungs, abscess in joints, aborted foetus, vaginal discharges or semen).

Epidemiological data have revealed a high diversity of serotypes among *S. suis* isolates, with serotype *cps*2 as the most associated with invasive diseases in humans [4, 14, 16, 31]; and serotypes 2, 9 and 14 are considered to be more invasive than others in pigs [14]. For *S. suis* serotype 2, it has been demonstrated that the capsule is an important virulence factor; however, unencapsulated *S. suis* strains might also invade host tissues, though to a lower degree [12]. The relevance of particular serotypes can vary geographically, even temporally. In China, *cps*2 accounts for more than 70 % of the systemic *S. suis* diseases in piglets [32], while in the Netherlands and Germany, *cps*9 emerged as the most common serotype responsible for a major fraction of invasive *S. suis* diseases of piglets [19]. On the other hand, *cps*7 strains have frequently been associated with bronchopneumonia in Scandinavia and Germany [11].

The identification of the *S. suis* SS13 *cps*2 isolate was confirmed by sequencing a fragment of the 16S rDNA gene and a fragment of *cps*2 gene. BLAST analysis of the sequences revealed a 99 % identity to *S. suis* type strain, which is consistent with the values expected for strains of a single species (99 % for 16S rDNA). The similarity level for the query sequence never exceeded 97 % with other *Streptococcus* species; these values consistent with distinct species in the same genus [25]. The phylogenetic relationship among the strains was shown with a tree (Figure 1), indicating a tight clustering of the SS13 isolate (JF266696) with known serotypes of *S. suis*. Other non-*S. suis* streptococcal strains were observed clearly separated from the *S. suis* cluster.

Multiple alignment of the nucleotide sequences of the *cps*2 fragment (JF266697) revealed that the sequence corresponded to strains of *S. suis* (Figure 2), only differing in one nucleotide change at position 836 (A→T), this mutation resulting in a possible amino acid variation from Lys_{279} by Ile_{279} at the C-terminus.

The 1492 bp PCR product corresponding to the suilysin gene was identified in three *cps*2 isolates from animals with pneumonia and only in one non-typable isolate from tonsils of an asymptomatic animal (Table 2). Suilysin is a pore-forming cholesterol-dependent cytotoxin. Though suilysin is not essential for virulence of *S. suis csp2* isolates in pigs, *in vitro* experiments suggest that it may have important functions for the interactions with host cells [5]. Tarradas *et al.* [33] detected suilysin production by *S. suis* isolated from diseased and healthy carrier pigs in Spain.

The 626 bp fragment of the gene encoding EF was detected only in two *cps*2 isolates. The EF was identified as an extracellular protein associated with virulence in serotypes 1, 2, 1/2, 14 and 15 [17]. The function of EF is still unknown, since isogenic *epf* mutants were as virulent as the wild-type in experimental infections, indicating that EF is associated but not essential for virulence [34].

The gene fragment encoding the MRP protein was amplified in *cps*2 isolates from cases of pneumonia and asymptomatic animals; this fragment was detected in the four *cps*9 isolates. The function of the 136 kDa MRP protein is unknown. It was discovered as a factor released from virulent *csp2* strains after muramidase treatment but expression of the MRP was found among strains of serotypes 1, 2, 1/2, 14 and 15 [17]. Different studies have shown that the presence of the gene did not always correlate with actual expression of the respective protein. Fittipaldi *et al*. [35] detected the *mrp* gene in 92 isolates of *S. suis* but the expression of MRP by Western blotting was only positive in 46 isolates, due, in most cases, to frameshift mutations in the 5′ end of the gene, resulting in premature stop codons. The effective production of the virulence markers by the tested isolates specifically in the genotype *cps*2$^+$ *mrp*$^+$ *epf*$^+$ *sly*$^+$ would be also verified.

As *mrp* seems to be a silent gene in some strains, it might also be discussed that phenotyping rather than genotyping should be performed in diagnostic laboratories [4]. Although not ideal, typing methods based on protein expression of these markers confirm the

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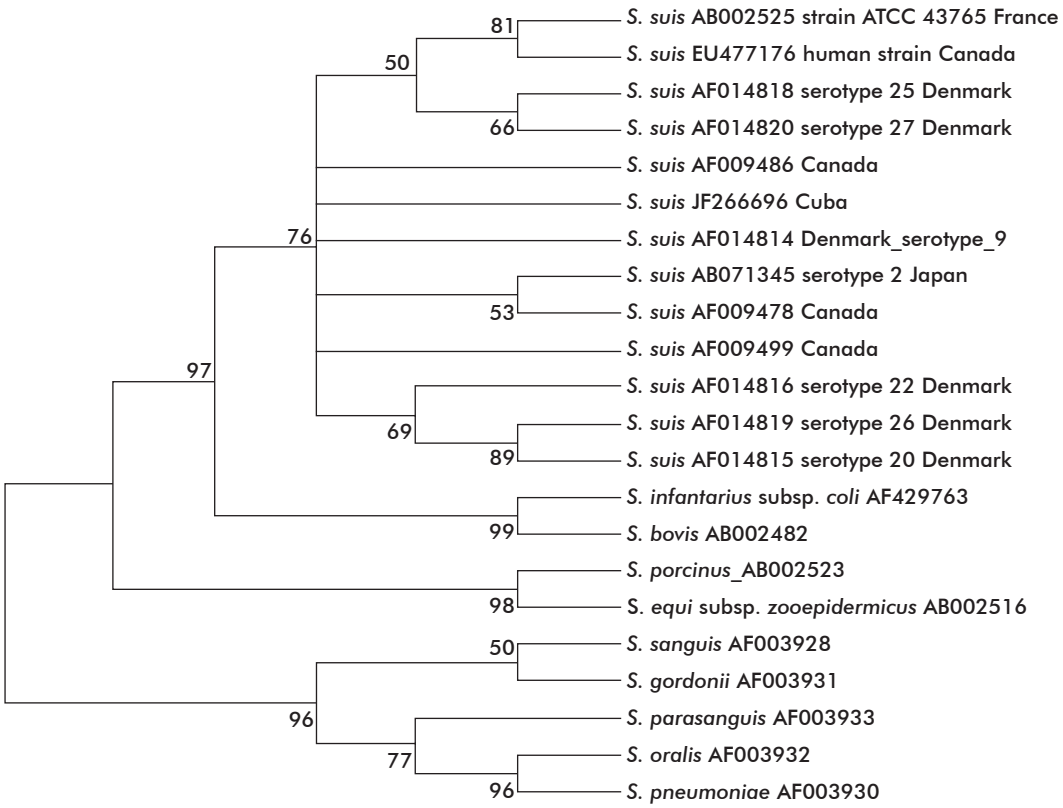

Figure 1. Phylogenetic tree of *S. suis* SS13 strain sequence (GenBank accession number JF266696), fourteen known serotypes of *S. suis* strains and tenth other streptococcal species with the genomic DNA fragment of the 16S rRNA. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.19159970 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA5.

phenotype because the PCR assays only detect a partial sequence of a gene. However, protein expression levels may be affected by many factors, as growth conditions, or even induced at specific stages of the infection process as it is known for many important, highly regulated virulence factors [36, 37]. The most promising candidate for improvement of *S. suis* diagnostics is the use of probes as the *ivs-25/iri-1*, which encodes a putative general virulence regulator. It discriminates between virulent and avirulent strains [37].

The analysis of the three virulence genetic markers showed the *cps*2^{+} *mrp*$^{-}$ *epf*$^{-}$ *sly*$^{-}$ as the most frequent genotype, in three isolates from abscesses of pigs with arthritis, one isolate from an aborted foetus and eight isolates from lungs. Only an isolate from lung showed the genotype *cps*2^{+} *mrp*$^{+}$ *epf*$^{+}$ *sly*$^{+}$. Despite the lack of evidence for a critical role of one or more of these putative virulence factors, they may nonetheless serve as virulence markers, since MRP, EF, and SLY are typical of Eurasian strains while they are almost absent among less virulent North American strains [1, 26, 35]. Strains with the *mrp*$^{+}$ *epf*$^{+}$ *sly*$^{+}$ genotype are known to be highly virulent, from European epidemiological studies [17] and pig experimental infections [4]. However, the percentage of *S. suis csp2* strains recovered from diseased pigs and the number of cases of human disease is lower in North America than in other parts of the world [36]. Therefore, it is very probable that the virulence of the Cuban *S. suis cps*2 isolates associated with invasive processes is similar to that reported for North American strains. Further studies with a larger number of Cuban isolates are required to definitively confirm this hypothesis.

Serotype determination remains a valuable tool used by veterinary practitioners and diagnosticians to understand the epidemiology of a particular outbreak and/or to increase the possibility for success of a vaccination program for a given herd [35]. The PCR assays used in this study can differentiate the three *cps* genotypes (2, 7 and 9) and additionally detect the important virulence associated factors genes *mrp*, *epf* and *sly* (Table 2). Taking into account methods for serotyping, the PCR assays are rapid, reliable, and sensitive. Moreover, these PCR assays could be used directly with tonsillar specimens from infected or carrier animals so that isolation of single colonies could be omitted. Therefore, these tests will undoubtedly contribute to a more rapid and reliable diagnosis of *S. suis* and may facilitate control and eradication programs [12].

Conclusions

So far, this is the first study on the molecular identification of *S. suis* isolates in Cuba from disease cases in pigs. *S. suis* isolates belonging to the capsular serotypes *cps*2 and *cps*9 were detected by genotyping

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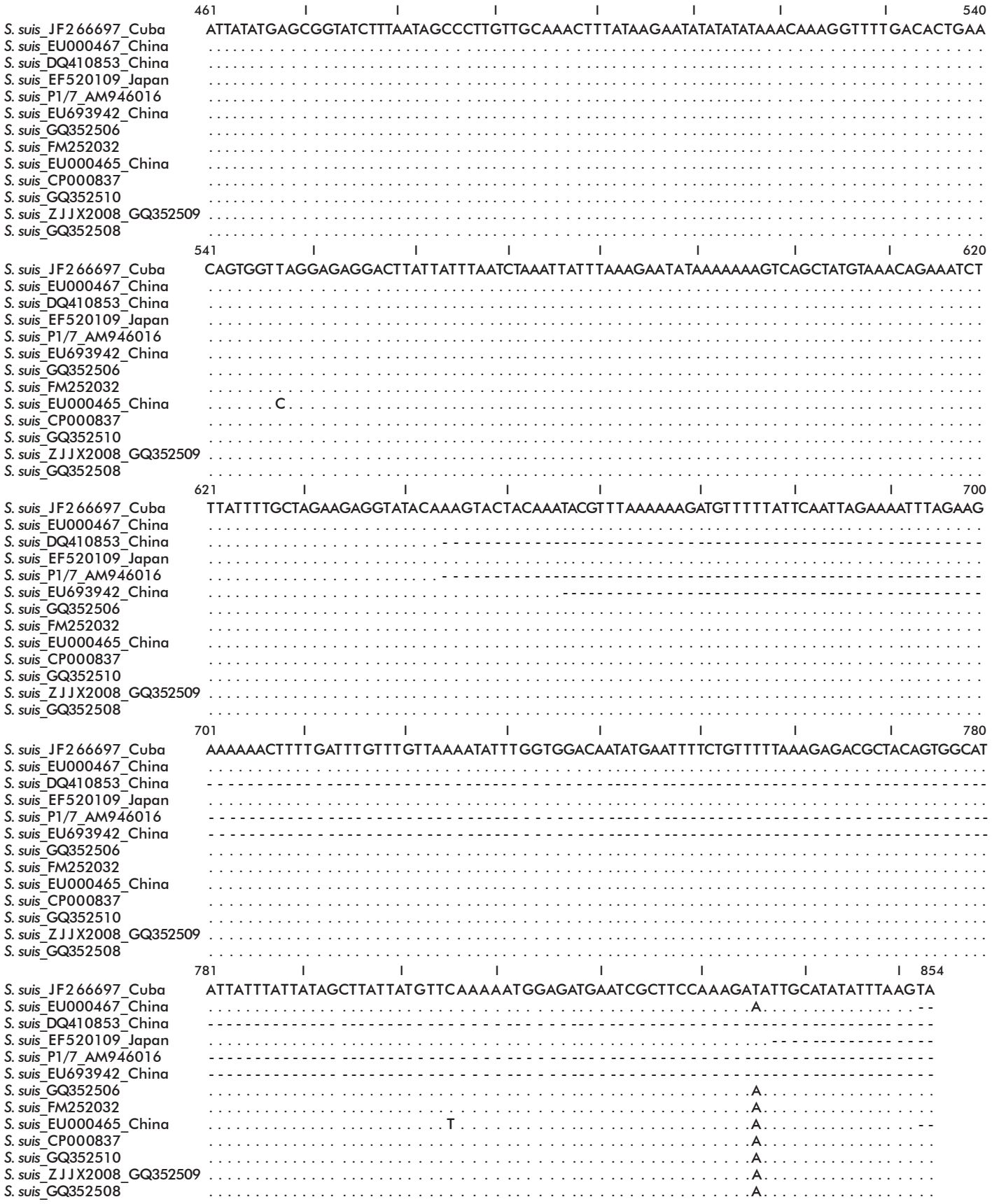

Figure 2. Alignment of the nucleotide sequences of the *cps2* fragment genes from *S. suis*. Sequence of the Cuban isolate (GenBank accession number JF266697) was taken as consensus. Identical bases are indicated by points and gaps inserted into the sequences are indicated by horizontal dots.

of the *cps2* and *cps9* genes, respectively. This is also the first report indicating the occurrence of at least two capsular types and nine different genotypes among the *S. suis* isolates tested. A larger scale sampling among pig farms from different Cuban regions could enable us to design a strategy to minimize the risk of exposure to *S. suis*.

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