



OPEN Development of a high-throughput MassARRAY-based single assay for the characterization of *Streptococcus suis* species and serotypes

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Streptococcus suis is a significant porcine pathogen and zoonotic agent responsible for infectious diseases in humans worldwide. It is classified into 29 serotypes, each with varying geographical prevalence and pathogenicity. Hence, serotyping of *S. suis* is crucial for active surveillance, outbreak monitoring, and infection control. This study developed a novel MassARRAY-based single assay to simultaneously identify *S. suis* species and differentiate all 29 serotypes. The assay targeted glutamate dehydrogenase (*gdh*) and recombination/repair protein (*recN*) for species identification, and capsular polysaccharide (*cps*) genes for serotyping. Based on single nucleotide polymorphisms (SNPs) at position 483 of *cpsK* gene, the assay accurately distinguished between two pairs of serotypes: ½ and 2, as well as 1 and 14. The assay, validated with genomic DNA from 105 whole-genome sequencing (WGS)-confirmed isolates, demonstrated 100% specificity and sensitivity for both species identification and serotyping. In the evaluation with 143 field isolates, the results demonstrated perfect agreement between the MassARRAY-based assay and WGS for species identification, with 100% sensitivity and specificity, and for serotyping, with 99.15% sensitivity and 100% specificity (κ -value = 0.98). The agreement between WGS-based serotyping and multiplex PCR serotyping was substantial (κ = 0.65). Moreover, the results showed that the limit of detection for both species and serotypes ranged from 1 to 10 pg of gDNA per reaction.

Keywords *Streptococcus suis*, MassARRAY assay, Mass spectrometry, MALDI-TOF, Serotyping

Streptococcus suis is a significant zoonotic pathogen primarily affecting pigs but also capable of causing severe infections in humans^{1,2}. *S. suis* exhibits considerable genetic diversity, characterized by multiple serotypes distinguished by variations in capsular polysaccharide (CPS) composition³. Over 29 serotypes have been identified to date with several non-typeable serotypes reported worldwide^{4,5}. The pathogenic potential and geographic distribution of *S. suis* serotypes vary, with each distinct serotype exhibiting diverse levels of bacterial virulence and unique patterns of host adaptation^{1,3}. Serotype 2, the most common and virulent type, is frequently recovered from diseased pigs and human clinical cases worldwide. In addition, other serotypes with an extensive reservoir of genetic diversity, such as serotype 5, 7, and 9 isolates, are becoming more of concern in different countries^{5,6}. Therefore, understanding serotype distribution is essential for analyzing epidemiological population dynamics, implementing effective control measures, and predicting and managing potential outbreaks.

In the serotyping of *S. suis*, the serological method is considered the gold standard. However, this approach has significant limitations for laboratories lacking access to the necessary antisera. It is also expensive, time-consuming for antisera preparation, and unable to readily discriminate between certain paired serotypes, such as ½ and 2 or 1 and 14, which exhibit cross-reactivity in the test⁷. Recently, PCR-based serotyping, including

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general and multiplex PCR techniques targeting serotype-specific *cps* genes, has been adopted as standard rapid methods for serotyping *S. suis* strains^{8,9}. Due to the similar gene content of their respective *cps* loci, these DNA-based methods still cannot differentiate between serotypes 1 and 14 and between serotypes ½ and 2, necessitating multiple tests for accurate identification. However, by targeting single nucleotide polymorphisms of *cpsK*, a PCR-mismatch mutation assay and a PCR-restriction fragment length polymorphism method have been developed to distinguish serotypes 1 from 14, as well as serotypes ½ and 2^{10–12}. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which relies on peptide mass fingerprinting (PMF), has also been applied for *S. suis* species identification^{13,14} and serotyping¹⁵. However, the accuracy and precision of the MALDI-TOF MS requires high quality and comprehensiveness of the reference PMF library. Since *S. suis* strains and serotypes exhibit significant geographical diversity, especially among pig-isolated strains, the PMF library must include the specific PMF profiles of *S. suis* prevalent in the region where the method is applied to ensure accurate classifications^{14,15}. Recent advances in whole-genome sequencing (WGS) technologies with a pipeline for in silico determination of *S. suis* serotype from short-read WGS data allows the sequencing of hundreds of bacterial genomes and identifying all 29 *S. suis* serotypes rapidly and relatively inexpensively¹⁶. However, this approach requires complex bioinformatic analysis and may not be available in resource-limited laboratories, making it still less accessible for routine surveillance work. Considering all these limitations, there is a clear need for a reliable and easily accessible method for *S. suis* serotyping, especially in surveillance efforts.

The advent of MassARRAY technology has proven to be a robust and adaptable approach for high-throughput genotyping and mutation detection in the realm of molecular biology and genetics research. The principle of MassARRAY technology involves the amplification of multiple targeted DNA sequences in a single reaction, simultaneously generating amplicons that serve as DNA templates for a single-nucleotide extension reaction. As a result, DNA products generated with specific molecular masses, are subsequently analyzed using mass spectrometer^{17,18}. This unique feature enables the identification and analysis of targeted genes based on their molecular masses, eliminating the need for chemical labelling and any PMF or WGS reference database for data analysis. Over the past decade, MassARRAY has proven its potency for both molecular research and applications in diverse fields such as cancer profiling¹⁹ and pathogen detection^{20–23}. The achievements of MassARRAY applications are underscored by its ability to deliver detailed and accurate results in the intricate landscape of microbial identification and characterization, making it a valuable tool in a wide range of research and diagnostics. In this study, we reported the development of a high-throughput MassARRAY-based single assay for the characterization of *S. suis* species and serotypes.

Results

Primer design and properties

In this study, MassARRAY-based multiplex-PCR targeting *gdh* and *recN* were used to species-specific identification of *S. suis*. The combination of 29 *cps* and *cpsK* loci was used to differentiate *S. suis* serotypes. Thirty primer sets were designed based on the conserved regions through the MassARRAY® Assay Design Suite software which provided the multiplex-PCR primer lengths of 29–34 bp with a melting temperature (*T*_m) range of 66.5–72.4 °C and %GC content of 38.2–51.6. These primers generated an amplicon length of primary PCR product varied from 119 to 225 bp. Based on the specific sequence selected, the software generated a set of extension primers (EP) (15–28 bp) with molecular mass ranged from 4,467.90 to 8,663.70 Da which allowed the production of distinct molecular masses of single-base extension (SBE) products, 4,715.10–8,990.80 Da. The primer properties were described in Table 1.

Validation of specificity and sensitivity of the developed MassARRAY-based assay

An optimal MassARRAY-based method was obtained by adjusting the oligonucleotide sequence of primers, FP, RP, and EP, optimizing the primary multiplex PCR and SBE reaction condition, and adjusting the amount of EP to allow a complete SBE reaction without any cross-reactivity with non-target pathogens.

The assay was optimized using purified gDNA from various control strains, including 30 *S. suis* with 29 serotypes and non-typeable strain, *S. suis* serotype 2 reference strain P1/7, seven other *Streptococcus* species (*S. agalactiae*, *S. anginosus*, *S. gallolyticus*, *S. orisratti*, *S. parasuis*, *S. pneumoniae*, and *S. pyogenes*), and eight other bacterial strains (*Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Enterococcus faecalis*, *E. faecium*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Staphylococcus aureus*). The chromatogram of negative control reactions without DNA template demonstrated unextended primer (UEP) peaks with expected molecular masses (Fig. 1A and Fig. S1) while the expected molecular mass of SBE products of *S. suis* *gdh* and *recN* were present in the reaction with *S. suis* serotype 2 reference strain P1/7 (Fig. 1B). No SBE products or unexpected spectra were observed in negative control reactions with non-*S. suis* *Streptococcus* spp. (Fig. S2) and non-streptococcal bacteria (Fig. S3). The result indicated a high capacity of the developed assay to distinguish *S. suis* from *S. suis*-like bacteria and other bacterial species.

The distinctive molecular mass of the SBE products allowed all 29 serotypes of *S. suis* to be completely distinguished under a single-assay platform (Fig. 2). Moreover, due to the designed EP specifically targeting a SNP at position 483 of *S. suis* *cpsK* gene, the SBE reaction could produce SBE products with different molecular mass. As shown in this study, when *cps* ½J/2J SBE product with a molecular mass of 5,940.90 Da were present, the distinct base calling of the *cpsK* SBE product at position 483 as G (8,820.80 Da) and T (8,844.80 Da) allowed for differentiation between serotypes 2 and ½, respectively. Similarly, in the presence of *cps* 1J/14J SBE products with a molecular mass of 6,491.10 Da, the base calling of the *cpsK* SBE product at position 483 as T (8,844.80 Da) and G (8,820.80 Da) indicated the presence of serotypes 1 and 14, respectively (Fig. 3).

The assay sensitivity was assessed using purified gDNA from *S. suis* serotype ½, 1, 2, 3, 5, 14, 19, and 27. These serotypes were selected as representative samples due to their significance in both humans and pigs, as well as their UEP molecular masses spanning across the chromatogram, providing a comprehensive evaluation of the

<i>S. suis</i> serotype	Primer name	PCR primer (FP, RP)		PCR amplicon (bp)	Extension primer (EP)		Molecular mass (Da)	
		Tm (°C)	GC (%)		Tm (°C)	GC (%)	UEP	SBE product
1, 14	cps1J/14J	68.4, 67.8	46.7, 43.3	132	50.0	45.0	6164.00	6491.10
½, 2	cps½/2J	69.1, 66.8	46.7, 41.4	168	49.1	50.0	5653.70	5940.90
3	cps3L	70.5, 68.1	50.0, 41.9	195	50.2	25.0	8663.70	8990.80
4	cps4M	70.5, 67.6	50.0, 43.3	156	49.0	29.2	7282.80	7570.00
5	cps5N	70.0, 70.9	46.7, 50.0	140	58.5	54.5	6652.40	6979.50
6	cps6I	70.3, 69.5	50.0, 46.7	177	47.6	44.4	5561.60	5888.70
7	cps7H	72.2, 70.6	50.0, 50.0	119	57.4	45.8	7244.80	7492.00
8	cps8H	69.3, 71.2	46.7, 50.0	149	50.4	40.0	6064.00	6311.20
9	cps9H	70.3, 70.5	45.2, 50.0	153	54.5	44.0	7740.10	8011.30
10	cps10M	69.2, 71.3	43.3, 50.0	155	49.9	56.2	4978.30	5265.50
11	cps11N	70.2, 71.4	50.0, 50.0	156	51.0	33.3	7311.80	7638.90
12	cps12J	69.5, 70.2	50.0, 45.2	149	46.7	47.1	5186.40	5473.60
13	cps13L	70.3, 69.8	46.7, 50.0	154	48.7	34.8	7136.70	7463.80
15	cps15K	69.1, 67.9	46.7, 38.7	140	47.0	18.5	8256.40	8527.60
16	cps16K	70.4, 70.6	50.0, 50.0	150	48.0	42.1	5683.70	5954.90
17	cps17O	71.9, 68.9	50.0, 43.3	162	53.0	58.8	5303.50	5574.70
18	cps18N	68.4, 68.9	43.3, 46.7	119	54.9	62.5	4914.20	5241.30
19	cps19L	69.2, 69.8	46.7, 46.7	134	54.0	66.7	4467.90	4715.10
21	cps21P	66.5, 68.8	41.4, 43.3	178	47.4	31.8	6718.40	6965.60
23	cps23J	70.5, 68.4	50.0, 43.3	187	50.8	30.8	7916.20	8187.40
24	cps24M	69.7, 69.6	46.7, 50.0	191	46.0	36.8	5907.90	6195.10
25	cps25M	69.6, 70.9	46.7, 50.0	182	48.0	35.0	6221.10	6548.20
27	cps27K	70.0, 71.2	46.7, 50.0	150	48.4	50.0	4842.20	5089.40
28	cps28L	69.0, 69.5	46.7, 43.3	144	47.4	31.8	6816.50	7063.70
29	cps29L	71.2, 71.8	50.0, 50.0	167	51.3	36.4	6761.40	7088.50
30	cps30I	69.2, 71.0	46.7, 50.0	132	56.0	42.3	8097.30	8424.40
31	cps31L	70.4, 68.0	46.7, 41.9	169	47.7	44.4	5526.60	5797.80
½, 1	cpsK	71.0, 69.4	50.0, 46.7	158	47.3	17.9	8573.60	8844.80
2, 14	cpsK	71.0, 69.4	50.0, 46.7	158	47.3	17.9	8573.60	8820.80
All <i>S. suis</i>	gdh	70.5, 68.9	45.2, 38.2	225	51.8	33.3	7409.90	7681.10
All <i>S. suis</i>	recN	69.8, 72.4	45.2, 51.6	191	55.3	43.5	7021.60	7348.70

Table 1. The information of primers designed.

assay performance. The purified gDNA was tenfold serial diluted and used as DNA templates for the analytical sensitivity determination. The result revealed the limit of detection (LOD) for the developed MassARRAY-based assay in the identification of species and serotypes of *S. suis* using gDNA samples, with an LOD of 10 pg for *S. suis* serotypes ½, 1, 2, 3, 5, 19, and 27, and 1 pg for *S. suis* serotypes 14 (Fig. S4).

The developed MassARRAY-based assay was validated using gDNA samples from 100 WGS-confirmed *S. suis* strains. The assay accurately identified *S. suis* species and classified the strains into 19 known serotypes, including serotypes 2 (n = 22), 8 and 29 (n = 10 each), 9 (n = 8), 3 and 21 (n = 7 each), 4 (n = 6), 16 (n = 5), 18 and 31 (n = 4 each), 1, 5, 7, and 28 (n = 2 each), 14, 15, 24, 25, and 27 (n = 1 each), whereas 4 remaining strains were non-typeable. When compared to WGS, the gold standard method in this study, the MassARRAY-based assay demonstrated 100% specificity and 100% sensitivity for *S. suis* species identification and serotype differentiation (Table S2). Furthermore, five WGS-confirmed *S. parasuis* strains were included in the assay validation as closely related species that had previously been misclassified as *S. suis* serotypes 20, 22, and 26²⁴. The results showed no positive mass spectra of SBE products for the *gdh*, *recN*, and *cps* genes, confirming that *S. parasuis* was correctly identified as non-*S. suis* (Table S2).

Evaluation of the developed MassARRAY-based assay performance with field samples

The performance of MassARRAY assay was evaluated using a total of 143 presumptive *S. suis* isolated from nasopharyngeal swab of slaughtered pigs. Based on WGS-based serotyping, 117 strains were categorized into 21 serotypes, including serotype 2 (n = 14), 8 (n = 13), 29 (n = 12), 3 (n = 10), 19 (n = 9), 4 (n = 8), 31 (n = 7), 5, 7, 18, and 24 (n = 6), 16 (n = 5), 28 (n = 4), 21 (n = 3), ½ (n = 2), 11, 12, 14, 15, 23, and 30 (n = 1 each), whereas 26 strains belonged to non-typeable (Table S3). Analysis using the MassARRAY-based assay correctly identified the species of all tested *S. suis* strains. However, one discrepancy in serotyping results was observed between the two assays; *S. suis* YM364 designated as serotype 21 by WGS-based serotyping was negative by MassARRAY-based assay. As a result, the MassARRAY-based assay demonstrated 99.15% sensitivity and 100% specificity, compared to WGS-

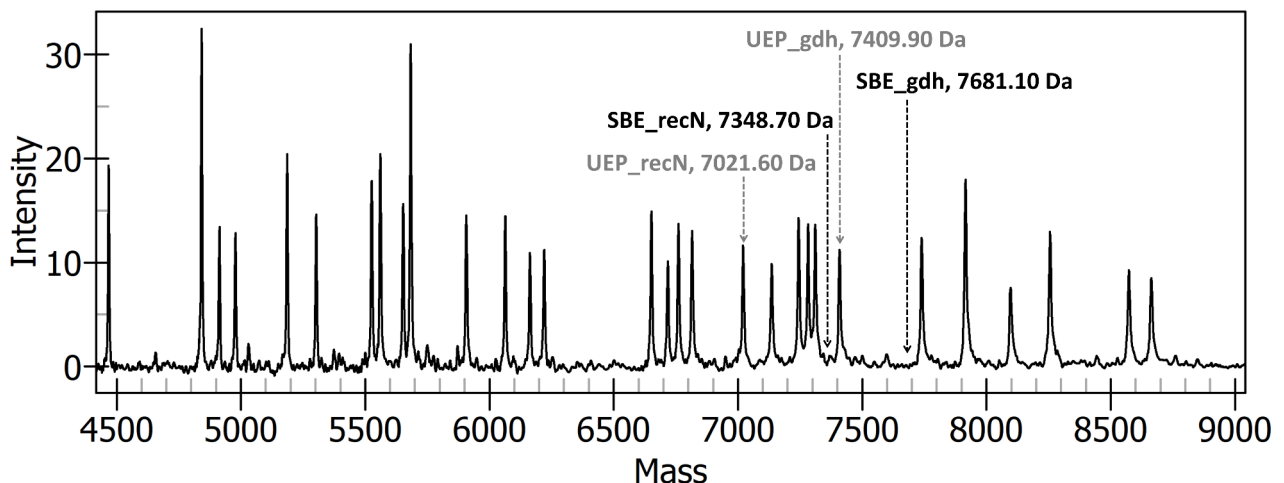
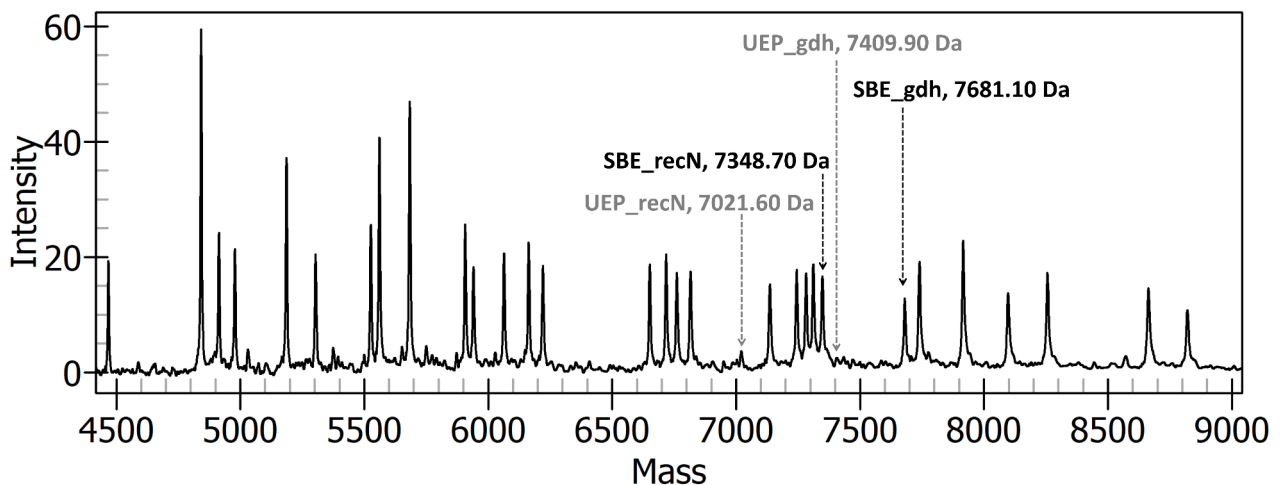
(A) Negative control**(B) *S. suis* serotype 2**

Fig. 1. Chromatograms illustrate the specific molecular mass of unextended primers (UEP) and single-base extension (SBE) products corresponding to target genes, *gdh* and *recN*. **(A)** demonstrates molecular mass of UEP in a negative control, no DNA template. **(B)** demonstrates molecular mass of SBE products in a positive control, *S. suis* serotype 2 reference strain P1/7. The x-axis represents the molecular mass and the y-axis represents the peak intensity.

based serotyping (Table 2). There was a high degree of agreement between the developed MassARRAY-based assay and WGS-based serotyping data with a κ -value of 0.98.

On the basis of multiplex PCR serotyping, 86.71% (124/143) of *S. suis* strains were correctly characterized, while the remaining 19 strains, including serotype 19 (n = 6), 3 (n = 5), 24 and 31 (n = 2 each), and 11, 15, 28, and 30 (n = 1 each), showed negative results and were interpreted as non-typable serotype (Table S3). Compared to the WGS-based serotyping data, the sensitivity and specificity of the multiplex PCR serotyping were 83.76% and 100%, respectively (Table 2). In addition, the agreement of *S. suis* multiplex PCR serotyping was also good with κ -value of 0.65. All 19 samples with discordant results were retested using simplex PCR reaction, yielding results consistent with the WGS-based serotyping. Consequently, combining the results obtained from multiplex and simplex PCR reactions, all 143 tested samples were in concordance with the WGS-based serotyping. However, it is important to note that the PCR-based serotyping is not capable of differentiating the two serotype pairs: 1/2 and 2, and 1 and 14.

Discussion

S. suis comprises distinct serotypes with geographical variation and some serotypes are clinically important pathotypes^{1,6}, leading to significant farm losses and economic burdens, as well as pose a threat to human health through zoonotic transmission^{1–3}. Effective methods for serotyping of *S. suis* are therefore crucial for the early detection and monitoring of *S. suis* pathogenic strains, which is essential for implementing prevention and control measures to protect both animal and human health.

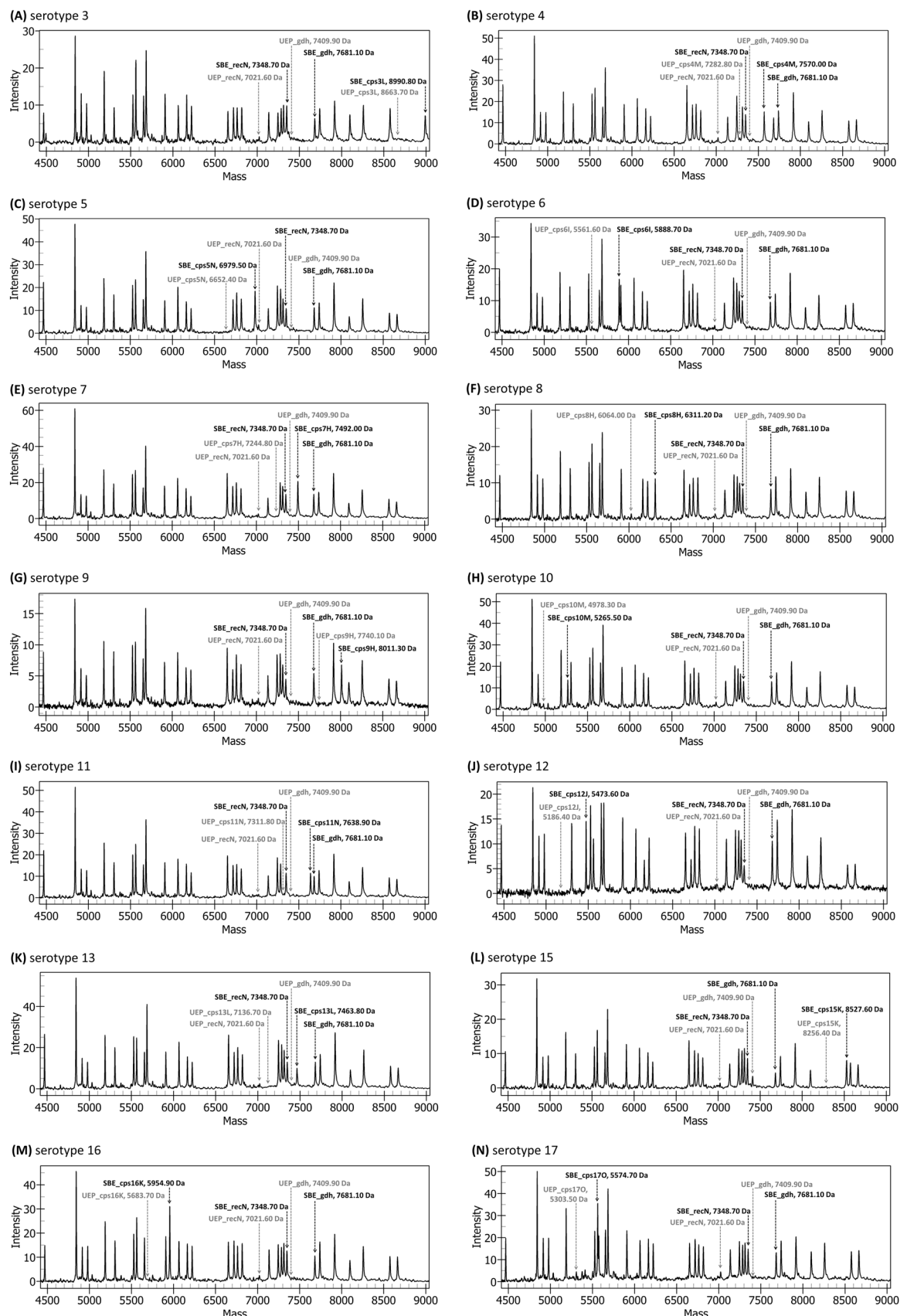


Fig. 2. Chromatogram illustrates the specific molecular mass of unextended primers (UEP) and SBE products (SBE) corresponding to target genes, *gdh*, *recN*, and *cps* of all 29 serotypes of *S. suis*. (A–K) serotype 3–13, (L–P) serotype 15–19, (Q) serotype 21, (R–T) serotype 23–25, and (U–Y) serotype 27–31. The x-axis represents the molecular mass and the y-axis represents the peak intensity.

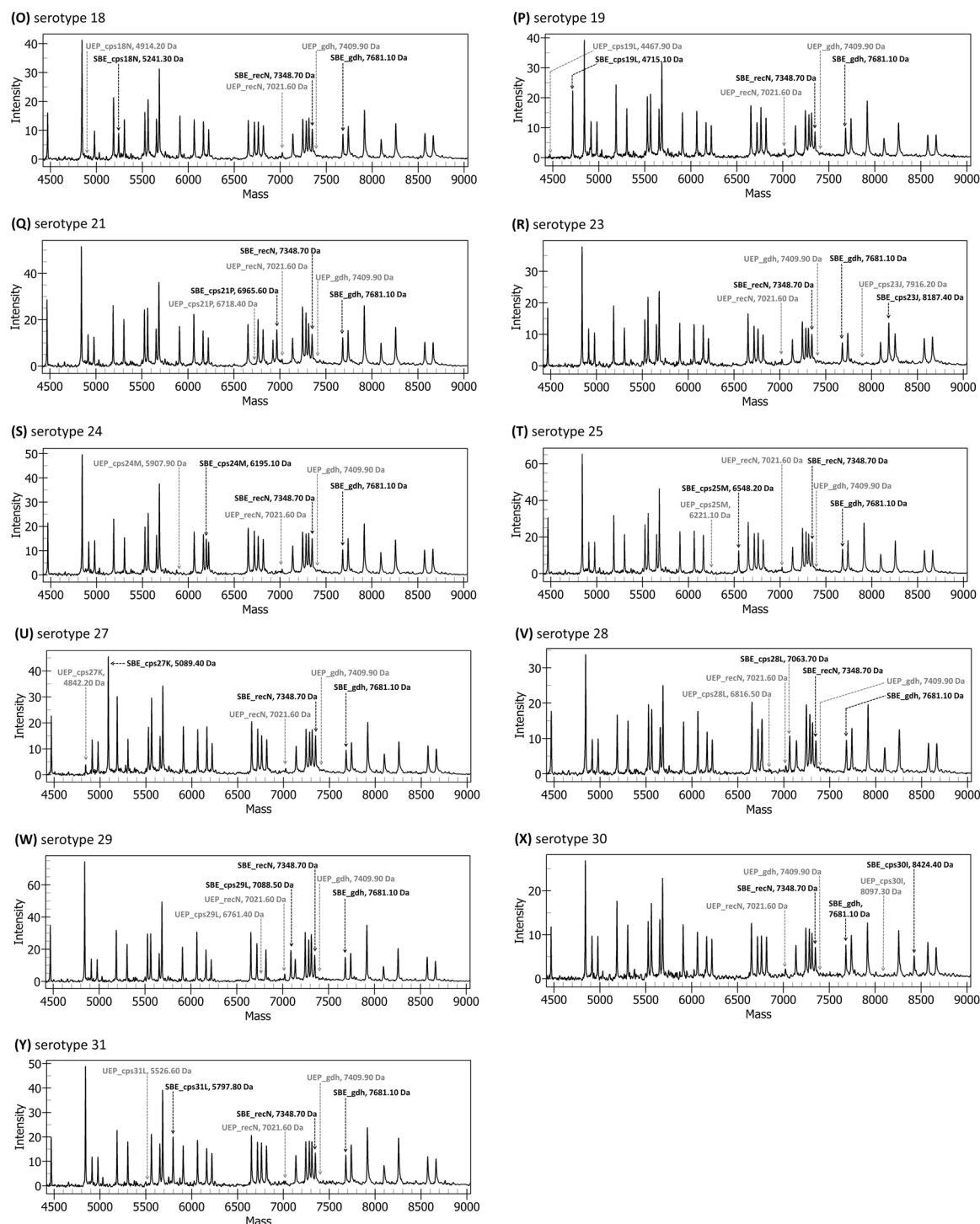


Figure 2. (continued)

To address the challenges of available methods for *S. suis* serotyping, in this study, a MassARRAY-based single assay was developed for simultaneously identifying *S. suis* species and serotypes. Unlike traditional serological methods, which rely on antigenic similarities, the MassARRAY approach focuses on unique sequences of genetic markers. By directly determining the distinct mass signatures of the SBE products of specified genes, including *gdh*, *recN*, and *cps*, in this study, the assay confirmed the presence of *S. suis* and helped overcome the difficulties in distinguishing closely related serotypes with similar antigenic profiles, such as *S. suis* serotypes ½ and 2, or 1 and 14.

Although the MassARRAY-based assay developed in this study offers significant advantages, including broad target coverage that enables the simultaneous detection of all *S. suis* serotypes in a single reaction, the MassARRAY technology presents certain challenges in laboratory settings. Notably, the turn-around time of

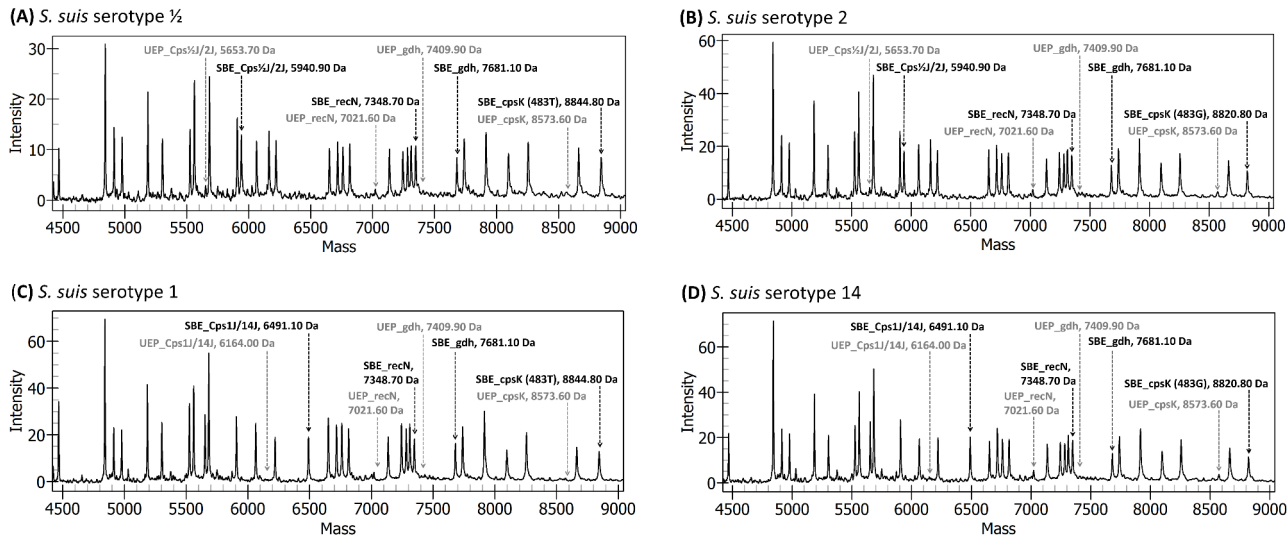


Fig. 3. Chromatograms illustrate the specific molecular mass of unextended primers (UEP) and single-base extension (SBE) products corresponding to target genes, *gdh*, *recN*, and *cps*. The presence of SBE products with molecular masses corresponding to the *gdh* and *recN* genes indicates the presence of *S. suis*. Meanwhile, distinct molecular masses of SBE products for the *cpsJ* and *cpsK* genes can differentiate between specific serotype pairs: serotypes 1/2 and 2, as well as serotypes 1 and 14. (A) *S. suis* serotype 1/2; (B) *S. suis* serotype 2 reference strain P1/7; (C) *S. suis* serotype 1; (D) *S. suis* serotype 14. The x-axis represents the molecular mass and the y-axis represents the peak intensity.

		WGS-based serotyping		Sensitivity (95% CI)	Specificity (95% CI)	Kappa (95% CI)
		Typeable	Non-typeable			
MassARRAY-based assay	Typeable	116	0	99.15 (95.33–99.98)	100 (86.77–100.00)	0.98 (0.93–1.00)
	Non-typeable	1	26			
Multiplex PCR	Typeable	98	0	83.76 (75.81–89.93)	100 (86.77–100.00)	0.65 (0.52–0.79)
	Non-typeable	19	26			

Table 2. The efficiency of *S. suis* serotyping methods in field isolates.

the MassARRAY technology spans several hours, and despite incorporating partial automation, it still requires multiple preparation steps and more hands-on time compared to conventional PCR methods. Moreover, the MassARRAY platform relies on pre-designed primers and assays targeting specific genetic markers, limiting its ability to detect unknown or novel bacterial strains^{22,23}. However, despite these technical limitations, its multiplexing capability, high specificity, and cost-effectiveness make it an attractive and valuable approach for large-scale *S. suis* surveillance and serotyping.

Compared to traditional PCR-based methods, the MassARRAY technology offers superior multiplexing capabilities in a high-throughput format without the need for labeling DNA^{17,18,20}. However, the main technical challenges lines in the design of oligonucleotide primers. Increasing the number of targets also introduces challenges in primer design, such as primer-dimer formation, heteroduplex formation, and primer competition, causing non-specific binding. To address these challenges, several hundred full coding DNA sequences of *S. suis* target genes, including *gdh*, *recN*, and *cps*, sourced from the NCBI database and in-house data collection of *S. suis* genomes isolated in Thailand, were used to obtain specific DNA templates, which were subsequently used for primer design using Assay Design Suite version 2.2 software. The specificity of each FP-RP pair was individually evaluated using simplex PCR reactions against gDNA from all serotypeable and non-typeable *S. suis* strains, non-*S. suis* *Streptococcus* spp., and non-*Streptococcus* bacterial species. The simplex PCR results demonstrated the highest specificity of 31 FP-RP pairs (data not shown). These primer pairs were then combined into a set of multiplex PCR primers, which was used in a MassARRAY single-assay panel.

In addition to its multiplex DNA amplification capability, the MassARRAY technology provides high specificity and sensitivity in genotyping assays through the SBE reactions. The mass of SBE products, which reflects the presence of SNPs and other genetic variations, can be directly analyzed using the MALDI-TOF mass analyzer^{17,18}. However, in the development of MassARRAY, consistent and clear visibility of mass intensity is critical for accurate genotyping and data interpretation. To achieve a uniform signal across all targeted analytes requires careful adjustment of the EP concentration used in the SBE reaction. In this study, the amount of each EP was calibrated according to the manufacturer’s recommendations to ensure the complete utilization of EP

in the SBE reaction, thereby balancing their amplification efficiency and ensuring that each target generated comparable peak intensity throughout the chromatogram.

Due to the large number of variable EP sequences and molecular masses, ionization efficiency during MALDI-TOF analysis can vary. This variability is inherent, as differences in the chemical structure of the oligonucleotides impact ionization differently¹⁸. Although this variability cannot be entirely avoided, it can be minimized by fine-tuning the concentration of each EP. In this study, before using the EP mixture in the SBE reaction, the peak intensity of all EP was thoroughly examined. In cases where outlier peaks were observed, the EP concentration was recalibrated by adding additional amounts of the specific EP into the EP mixture to balance the peak intensity across all EP peaks. By achieving this balance, the reliability and accuracy of the MassARRAY analysis were significantly enhanced, allowing precise characterization of the targeted SBE products.

Accurate bacterial species identification is essential for confirming diagnoses and disease surveillance. The developed MassARRAY-based assay effectively distinguished *S. suis* from the closely related species, *S. parasuis*, which had previously been misclassified as *S. suis* serotypes 20, 22, and 26²⁴. These findings highlighted the robustness of the MassARRAY-based assay in preventing misidentification and its consistency with the updated taxonomic classification of these species.

This study demonstrated the high specificity and sensitivity of the developed MassARRAY-based assay, with a LOD ranging from 1 to 10 pg of purified gDNA (equivalent to 4×10^2 – 4×10^3 copies) per reaction. It is important to note that the LOD assessment was conducted using representative *S. suis* strains, rather than all serotypeable strains. These representative strains exhibited a range of molecular masses, including the lowest (4,467.90 Da) and highest (8,990.80 Da), and varied %GC content (25%–67%). Although not all strains were tested, the data obtained from these representative strains could provide an indication of the overall performance of the developed assay.

The performance of the developed MassARRAY-based assay was evaluated using presumptive isolates, which included all serotypeable strains except for serotypes 1, 6, 9, 10, 13, 17, 25, and 27, as these serotypes were not detected in our field sample collection. While high-quality gDNA prepared through rigorous methods, resulting in very pure samples was used for WGS experiments, a simple heat-lysis procedure was chosen for gDNA extraction in the MassARRAY-based assay to streamline sample preparation, making the sample preparation process quicker and easier to handle, while also being compatible with downstream automated systems. However, the matrix in the crude extracts may interfere with the sensitivity and specificity of the assay reaction, particularly with the high complexity of primers used. As shown in this study, one discrepancy was observed in the serotyping results of sample YM364 between the developed MassARRAY-based assay and WGS-based serotyping. While WGS-based serotyping identified the sample as serotype 21, the MassARRAY-based assay yielded no base-calling result for the serotype, interpreting it as non-typeable strain. However, a simplex PCR using the *cps*21P FP-RP pair revealed a positive PCR reaction, reconfirming serotype 21, consistent with WGS-based serotyping result.

The same crude extracts were also used for serotyping via a multiplex PCR method⁸, in which the reaction involved less primer complexity than the multiplex PCR reaction used in the MassARRAY-based assay. Despite this, more discrepancies were observed in multiplex PCR-based serotyping compared to WGS-based serotyping than in the MassARRAY-based assay. When tested with simplex PCR using serotype-specific primers, all 19 non-typeable isolates identified by the multiplex PCR-based serotyping were determined to be typeable strains, consistent with WGS-based serotyping results. Genome analysis of the *cps* genes revealed no mutations at the primer binding sites, indicating no mismatches between the *cps* target sites and the primers. These findings highlight the importance of sample quality in ensuring the accuracy of serotyping results in both multiplex PCR-based serotyping and MassARRAY-based assay.

Both MALDI-TOF MS and MassARRAY share key similarities in being MS-based analysis, offering rapid, high-throughput platforms, broad bacterial identification capabilities, and cost-effectiveness in the long term^{15,20,21}. However, in mixed samples, the complexity and overlap of PMF may pose challenges for data interpretation in MALDI-TOF MS analysis^{14,15}. In contrast, DNA amplification of specific targets in MassARRAY can provide clearer and more specific mass spectra, facilitating easier data interpretation^{20,21}.

Overall, when using presumptive isolates from blood agar plates, the developed MassARRAY-based assay accurately identified both the species and serotype of *S. suis* by targeting specific genetic markers, resulting in highly accurate results. The performance of the developed MassARRAY-based assay was quite satisfactory, with a sensitivity of 99.15% and a specificity of 100%. However, applying this assay for direct use with field samples might be more challenging due to the potential overgrowth of environmental bacteria, which can hinder the detection of *S. suis*. Therefore, the use of fresh samples and suitable sample preparation are crucial to ensure accurate detection.

Conclusions

The developed MassARRAY-based assay, combining multiplex PCR with MALDI-TOF MS technology, offered an effective and reliable method for classifying and differentiating *S. suis* at both the species and serotype levels, covering all 29 serotypes. This assay demonstrated high precision without cross-reactivity with other *Streptococcus* species and accurately distinguished between *S. suis* serotypes ½ and 2, as well as serotypes 1 and 14. Furthermore, it allowed for rapid and high throughput testing of numerous samples in a single experiment. Given its high performance, this single-assay platform has the potential to become the method of choice for *S. suis* active surveillance systems, enhancing control strategies for *S. suis* infections.

Materials and methods

Bacterial and genomic DNA samples

Thirty *S. suis* control strains, which had been previously confirmed by whole genome sequencing (WGS), were representative of 29 different *S. suis* serotypes (serotype ½, 1–19, 21, 23–25, 27–31) and non-typeable serotype, along with *S. suis* serotype 2 reference strain P1/7 and other *Streptococcus* species, including type strains of *S. agalactiae* ATCC13813, *S. anginosus* ATCC33397, *S. gallolyticus* 55–1713, *S. orisratti* SS-391, *S. parasuis* SS-551, *S. pneumoniae* ATCC49619, and *S. pyogenes* WHO-28, were used as reference strains for MassARRAY assay development and optimization experiments (Table S1). Reference genomic DNA (gDNA) of other bacterial strains (n = 8), including *C. jejuni* MK7, *C. perfringens* WAL-14572, *E. coli* B171, *E. faecalis* TX0104, *E. faecium* TX0133a04, *L. monocytogenes* F6900, *S. typhimurium* 14,028, and *S. MRSA131*, were also used as negative control for assay development experiments (Table S1).

In addition, gDNA of 100 clinical *S. suis* strains isolated from diseased pigs, including 96 strains with different serotypes, and 4 non-typeable strains, and 5 strains of *S. parasuis* as negative controls, previously confirmed by WGS, were selected from our collection for assay validation (Table S2).

A collection of 143 presumptive *S. suis* strains with alpha-hemolysis colonies, Gram-positive staining, and catalase-negative results, that were retrieved from nasopharyngeal swabs of slaughtered pigs and confirmed as specific serotypes by WGS-based serotyping, were used as field samples to evaluate the performance of the developed assay (Table S3).

Preparation of genomic DNA

Streptococcus spp. was grown on Columbia blood agar (Thermo Fisher Scientific, USA) at 37 °C, in 5% CO₂ incubator for 18–24 h. The pure colonies were then used for gDNA preparation by using the cetyltrimethylammonium bromide (CTAB) method²⁵. The gDNA purification was conducted using DNA Clean & Concentrator kit (Zymo research, USA), following the manufacturer's instructions. The quality of all processed samples was verified using the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The purified gDNA with a minimum concentration of 40 ng/μL, OD260/OD280 and OD260/OD230 ratio of ≥ 1.8 were used for further downstream experiments.

A simple heat-lysis procedure was employed for gDNA extraction of the presumptive *S. suis* isolated from slaughtered pigs. A few bacterial colonies were selected from Columbia blood agar plate (Thermo Fisher Scientific, USA) and resuspended in 50 μL of distilled water. Cell suspension was subjected to boiling at 100 °C for 10 min and then frozen at –20 °C for 20 min. After centrifugation at 6,000 rpm at 25 °C for 5 min, a clear supernatant was subsequently used as the DNA template for evaluation of MassARRAY assay.

Serotype analysis by whole genome sequencing and multiplex PCR

The gDNA samples of *S. suis* strains were submitted to the Azenta GENEWIZ™ sequencing facility (Suzhou, China) for whole genome sequencing using Illumina HiSeq PE250 system (Illumina, USA). Raw paired-end reads were assembled de novo using SPAdes genome assembler through the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) service v3.6.2 (<https://www.bv-brc.org>) with default parameters. The bacterial strains were confirmed as *S. suis* using the criteria of ≥ 95% nucleotide identity to the *S. suis*-specific recombination/repair protein (*recN*) sequence. The average nucleotide identity (ANI) between the isolates and the reference genome, *S. suis* BM407 (GenBank accession no. NC_012926.1), was also calculated by FastANI v1.3 on Galaxy platform (<https://galaxytraker.org/>) with cut-off of ≥ 94% identity¹⁴. The serotype of the strains was further confirmed by the occurrence of the traditional 29 *cps* loci and a single-nucleotide substitution at the position 483 of the *cpsK* locus differentiating two serotype pairs, serotype ½ or 2 and 1 or 14, using the Center for Genomic Epidemiology's MyDbFinder 2.0 (<https://cge.food.dtu.dk/services/MyDbFinder/>).

PCR-based identification using species-specific primers targeting the *recN* gene was employed to identify *S. suis* isolates, following the method previously described by Lunha et al.²⁶. PCR reactions were conducted in a 10 μL reaction mixture, containing 0.5 μM of primers, 1X of Standard *Taq* buffer with 1.5 mM MgCl₂ (New England Biolabs, USA), 0.2 mM dNTP mix (Thermo Fisher Scientific, USA), 1 unit of *Taq* DNA polymerase (New England Biolabs, USA), and 10 ng of gDNA. The following PCR condition was used: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 54 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 7 min.

The serotypes of *S. suis* strains were determined using a multiplex PCR method⁸, comprising four independent PCR reactions targeting specific serotypes; (i) serotypes ½, 1, 2, 3, 7, 9, 11, 14, and 16, (ii) serotypes 4, 5, 8, 12, 18, 19, 24, and 25, (iii) serotypes 6, 10, 13, 15, 17, 23, and 31, and (iv) serotypes 21, 27, 28, 29, and 30 (Table S4). The 10 μL-volume of mixture contained the following components: 0.2 μM each of primers, 0.5 mM dNTP mix (Thermo Fisher Scientific, USA), 1X standard *Taq* buffer (New England Biolabs, USA), 1 unit of *Taq* DNA polymerase (New England Biolabs, USA), and 10 ng of gDNA. The reaction conditions for the multiplex PCR were as follows: an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 20 s, 62 °C for 1 min and 30 s, and 72 °C for 5 min.

Primers and assay design

Specific sequences of glutamate dehydrogenase (*gdh*) and *recN* genes were selected as genetic markers for designing species-specific primers for differentiating *S. suis* species from *S. suis*-like bacteria and other bacterial species. The *cps* genes of *S. suis* were used as DNA targets for serotype-specific detection. SNPs at position 483 of the *S. suis cpsK* gene were employed to differentiate between two pairs of serotypes: ½ and 2, and 1 and 14.

To design the MassARRAY-based assay, the full DNA coding sequences of target genes, including *gdh* (n = 235), *recN* (n = 231), and *cps* (n = 250), were retrieved from the National Center for Biotechnology Information (NCBI) genome database and aligned along with an in-house *S. suis* whole genome database (n = 225, unpublished data).

The multiple-sequence alignment was performed using a CLUSTAL-W alignment tool²⁷. The highly conserved and specific regions of individual targeted genes were subsequently chosen for the primer and assay design using Assay Design Suite version 2.2 software, following the manufacturer's recommendations (Agena Bioscience, USA).

The primer design process adhered to stringent parameters to minimize primer-dimer formation, hairpin loops, and false priming, ensuring the production of high-quality and specific primers. The specificity of designed primers was evaluated using the Basic Local Alignment Search Tool (BLAST) to ensure the highest level of primer specificity was achieved. Subsequently, each primer pair (forward and reverse primers, FP and RP) and a set of multiplex primers were rigorously evaluated using conventional PCR reaction with targeted and non-targeted gDNA samples, as well as a no template control to ensure primer specificity and absence of cross-PCR reactions. It is important to highlight that primer sequence and length were manually adjusted as necessary to achieve uniformity of melting temperature (T_m) and guanine-cytosine content (%GC) values among multiplex primers, facilitating PCR optimization. In each assay, FP and RP were used to amplify the template and extension primers (EP) were used for single-base extension (SBE) reaction. To avoid interference in the mass spectra, a 10-nucleotide tag (ACGTTGGATG) was added to the 5' end of amplification primers. All PCR amplification and extension primers were synthesized by Integrated DNA Technologies (Singapore).

Optimization of MassARRAY protocol and analysis

The MassARRAY-based assay optimization was performed using purified gDNA of 46 bacterial strains, including *S. suis* with 29 different serotypes and non-typeable serotypes ($n=30$), *S. suis* serotype 2 reference strain P1/7, other *Streptococcus* species ($n=7$), and other bacterial strains ($n=8$) (Table S1). The assay was optimized and modified under the manufacturer's recommendation.

A set of multiplex PCR primers consisting of 2 pairs of species-specific primers, *gdh* and *recN*, and 28 pairs of serotype-specific primers targeting 27 *csp* and *cpsK* genes was employed in multiplex PCR reaction. The reaction was carried out in a total volume of 5 μ L comprising 1X standard *Taq* buffer (New England Biolabs, USA), 0.25 mM of dNTPs (Thermo Fisher Scientific, USA), 0.1–0.2 μ M each of PCR primer, and 1 unit of *Taq* DNA polymerase (New England Biolabs, USA), and 10 ng of gDNA template. The PCR cycling conditions included an initial denaturation at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 65 °C for 2 min, and a final extension step at 65 °C for 5 min. The reaction was then terminated by cooling at 4 °C. Following the DNA amplification, the excess dNTPs were eliminated by adding 2 μ L of shrimp alkaline phosphatase (SAP) cocktail containing 0.073 units of SAP enzyme in 0.24X of SAP buffer (iPLEX® Pro, Agena Bioscience, USA). The dephosphorylation reaction was taken place in a final reaction volume of 7 μ L at 37 °C for 40 min, followed by inactivation at 85 °C for 5 min.

After the dephosphorylation reaction, the SBE reaction was performed according to the iPLEX® Pro and Gold Reagents User Guide (Agena Bioscience, USA). A mixture of EP was adjusted using regression method according to the manufacturer recommendation. Two microliters of the iPLEX extension cocktail, containing 0.142 units of the iPLEX enzyme, 0.222X of the iPLEX terminator nucleotide mixture, 0.47–2.08 μ M of the adjusted EP, and 0.222X of iPLEX buffer (iPLEX® Pro, Agena Bioscience, USA), was added to the SAP-treated PCR product. The SBE reaction was carried out in a final reaction volume of 9 μ L with an initial incubation at 95 °C for 30 s, followed by 40 cycles of one step denaturation at 95 °C for 5 s with five sub cycles of an annealing step at 52 °C for 5 s, a denaturation step at 80 °C for 5 s, and a final extension at 72 °C for 3 min. Subsequently, the SBE products were cleaned up by diluting with 29 μ L of sterile distilled water and desalted by conditioning with 13 μ L of a commercially available resin (Agena Bioscience, USA), according to the manufacturer's instruction before being analyzed. After exchanging salts with resin, the conditioned SBE products were dispensed onto a 96-well spectroCHIP using the MassARRAY Nanodispenser RS1000 and then measured the molecular mass by the mass spectrometer (MS). If an EP incorporated a terminator nucleotide, a spectrum representing the base-extended primer appeared and subsequently was identified by its expected molecular mass. The molecular mass and base calling data were analyzed and interpreted using TyperAnalyzer® software version 4.0.25.73 (Agena Bioscience, USA).

Determination of the detection limit

The MassARRAY-based assay detection limit was determined using purified gDNA from *S. suis* strains serotype ½, 1, 2, 3, 5, 14, 19, and 27, as representative samples which covered significant serotypes and various EP mass ranges. For each sample, gDNA concentration was adjusted into 5 ng/ μ L, serially tenfold diluted, and 2 μ L of each dilution were subsequently used as DNA templates. The lowest DNA concentration that provided positive result was considered as a detection limit.

Validation of MassARRAY-based assay

To assess the validity of this developed MassARRAY-based assay, the purified gDNA of 100 strains of *S. suis* and 5 strains of *S. parasuis*, with known genotype diversity as indicated by their genome sequence data, were used as samples (Table S2). The MassARRAY-based assay for *S. suis* genotyping was tested across multiple testing batches of each sample to confirm the precision and reproducibility of the results. The performance of the developed MassARRAY-based assay was assessed by comparing the results with those obtained from genome sequencing and multiplex PCR.

Evaluation of the MassARRAY-based assay performance with filed samples

The developed MassARRAY-based assay was evaluated using the collection of 143 presumptive *S. suis* isolated from nasopharyngeal swab of healthy slaughtered pigs in Nakhon Pathom Province, Thailand, during April 2022–February 2023 (Table S3). The alpha-hemolysis colonies of presumptive *S. suis* strains on Columbia

blood agar (Thermo Fisher Scientific, USA) were selected for gDNA extraction by simple boiling method and subsequently used as a DNA templates. The results were compared to those obtained from genome sequencing and multiplex PCR.

Statistical analysis

The sensitivity and specificity were calculated using the MedCalc Software Ltd. Diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic_test.php, version 22.026, accessed August 10, 2024). In addition, agreement analyses with Kappa (κ) results were performed using the publicly available GraphPad Prism web calculator (<https://graphpad.com/quickcalcs/kappa2/>, accessed August 10, 2024). Level of agreement was interpreted from Kappa values as previously described; no agreement ($\kappa < 0$), slight agreement ($0 \leq \kappa \leq 0.20$), fair agreement ($0.21 \leq \kappa \leq 0.40$), moderate agreement ($0.41 \leq \kappa \leq 0.60$), substantial agreement ($0.61 \leq \kappa \leq 0.80$), and almost perfect agreement ($0.81 \leq \kappa \leq 1.00$)²⁸.

Data availability

The datasets generated and/or analysed during the current study are available under BioProject accession number PRJNA1182519 at the National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/bioproject/1182519>.

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Author contributions

SJ (Co-corresponding Author): Methodology (design and development), Investigation, Data collection, Formal analysis, Writing–original draft, Review & Editing; KL (Co-corresponding Author): Investigation, Data collection, Formal analysis, Writing–Review & Editing, Funding acquisition, WC: Investigation, Data collection, Writing–Review & Editing; NM: Resources, Review & Editing; AK: Resources, Review & Editing, Critical revision; SY (Corresponding Author): Conceptualization, Supervision, Funding acquisition, Writing–original draft, Review & Editing, and Final approval of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

The experimental procedures were conducted according to the IBC Protocol Number: BT-IBC 052/64 at National Science and Technology Development Agency, Thailand. Ethical approval for animal care and use (Ethical Approval ID: ACKU66-VTN-006) was obtained from Kasetsart University, Thailand.

Additional information

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