

Exploring target-specific primer extension in combination with a bead-based suspension array for multiplexed detection and typing using *Streptococcus suis* as a model pathogen

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Abstract. We investigated the feasibility of an assay based on target-specific primer extension, combined with a suspension array, for the multiplexed detection and typing of a veterinary pathogen in animal samples, using *Streptococcus suis* as a model pathogen. A procedure was established for simultaneous detection of 6 *S. suis* targets in pig tonsil samples (i.e., 4 genes associated with serotype 1, 2, 7, or 9, the generic *S. suis* glutamate dehydrogenase gene [*gdh*], and the gene encoding the extracellular protein factor [*epf*]). The procedure was set up as a combination of protocols: DNA isolation from porcine tonsils, a multiplex PCR, a multiplex target-specific primer extension, and finally a suspension array as the readout. The resulting assay was compared with a panel of conventional PCR assays. The proposed multiplex assay can correctly identify the serotype of isolates and is capable of simultaneous detection of multiple targets in porcine tonsillar samples. The assay is not as sensitive as the current conventional PCR assays, but with the correct sampling strategy, the assay can be useful for screening pig herds to establish which *S. suis* serotypes are circulating in a pig population.

Key words: Bead-based suspension array; multiplex PCR; pigs; serotyping; *Streptococcus suis*; target-specific primer extension.

Introduction

PCR is a valuable technique that is widely used in diagnosis and control of animal disease.⁸ However, PCR and real-time PCR are limited in the number of assays that can be performed in one simultaneous reaction. For multiplex detection of nucleic acid sequences, parallel real-time PCRs on microfluidic arrays are possible,¹⁷ as are comprehensive syndromic microarrays,¹⁵ but both require costly arrays and equipment. Inexpensive microarrays that can be analyzed with inexpensive equipment also exist,^{4,11} but these assays depend on a fixed probe configuration. Multiplexing can also be set up as a bead-based suspension array for which the probe configuration is not fixed and the required equipment can be acquired at modest cost. A great advantage of bead-based arrays is that a large number of simultaneous assays are possible (i.e., 50, 80, or 500 depending on the equipment used). For these suspension arrays, several assay types are possible, for example hybridization of amplicons upon exponential amplification, or hybridization of single-stranded products upon linear amplification.^{6,10} The feasibility of target-specific primer extension (TSPE) combined with a suspension array was demonstrated for multiplexed detection of plant pathogens.^{30,31} We chose a similar approach to investigate multiplexed

detection of veterinary targets in samples of animal origin, using *Streptococcus suis* as a model pathogen.

S. suis is a significant swine pathogen in nearly all countries with an intensive pig industry, and also has considerable zoonotic potential.^{21,27,35} Over 30 different capsular serotypes of *S. suis* have been reported.^{3,19} Although serotype 2 is the most prevalent serotype worldwide in diseased pigs,¹² serotypes 1, 7, and 9 are also frequently found to be associated with diseased pigs.³⁸ Good laboratory assays to identify *S. suis* carrier pigs are required to better understand the epidemiology of the disease and to facilitate effective control measures. Many multiplex PCR assays for the detection and (sero)typing of *S. suis* in clinical samples have been described, but these often rely on enriched samples^{22,33,36} or on isolates, as reviewed previously.¹² To date, no complete

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S. suis detection and typing assay has been reported that can be used on DNA isolated from clinical samples to our knowledge.

We set the detection of 6 targets by their nucleic acid sequences as the objective. The selected targets are 4 serotype-specific genes for *S. suis* serotypes 1, 2, 7, and 9, one serotype-independent generic *S. suis* gene encoding glutamate dehydrogenase (*gdh*), and a virulence marker encoding the extracellular protein factor (*epf*). For the latter, a design was made that exclusively targets the virulent form that has no inserts.²³ We compared the resulting assay (i.e., a combination of a multiplex PCR, a TSPE, and a suspension array) with a panel of conventional PCRs, and determined the strengths and weaknesses of the proposed assay.

Materials and methods

Strains and culturing

Reference strains of all *S. suis* serotypes¹⁹ were used, complemented with a serotype 2 field strain (strain 3)³² that is positive for extracellular protein factor (EF+). Further, 31 non-*S. suis* bacteria from an in-house collection³⁶ were used (*Actinobacillus pleuropneumoniae*, *Bacillus subtilis*, *Bordetella bronchiseptica*, *Campylobacter coli*, *Enterococcus faecalis*, *Erysipelothrix rhusiopathiae*, *Escherichia coli*, *Haemophilus parasuis*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Micrococcus* spp., *Pasteurella multocida*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Staphylococcus hyicus*, *S. bovis*, *S. dysgalactiae*, *S. dysgalactiae* subsp. *equisimilis*, *S. gordonii*, *Streptococcus* group L, *S. milleri* III, *S. mitis*, *S. mutans*, *S. oralis*, *S. pneumoniae*, *S. pyogenes*, *S. sanguinis*, *S. uberis*, *S. zooepidemicus*, *Yersinia enterocolitica*). Bacteria were grown overnight at 37°C in air with 5% CO₂ on 6% horse blood agar plates (Columbia blood agar base, Oxoid, Landsmeer, The Netherlands) or on heart infusion agar plates containing 5% sheep blood and 0.1% nicotinamide adenine dinucleotide (Biotrading, Mijdrecht, The Netherlands; *A. pleuropneumoniae* and *H. parasuis*).

Clinical samples

A farrow-to-finish piggery that had recent problems related to *S. suis* infections was selected. Thirty 8-wk-old pigs and 10 farrowing sows were sampled by tonsillar brushing using a sterile tooth brush.²⁸ Immediately after sampling, the tooth brushes were transported on ice to the laboratory in 15 mL of Todd-Hewitt broth (Oxoid) with 0.25% *Streptococcus* selective supplement SR0126 (Oxoid) and 0.2 mg/mL of crystal violet. The tubes were vortexed 5 times for 30 s, and placed for 1 h in an ultrasonic water bath at room temperature. A 1.5-mL aliquot was stored at -80°C in the presence of 15% glycerol.

DNA preparations

DNA of all strains was isolated using a phenol-chloroform extraction method as described.⁹ DNA of *S. suis* serotypes 1, 2, 7, and 9 reference strains was prepared using the MagNA Pure LC total nucleic acid isolation kit and MagNA Pure LC isolation robot (Roche Diagnostics, Almere, The Netherlands), according to the instructions of the manufacturer. To obtain DNA from the porcine tonsillar tooth brushes, a 200-μL subsample from the stored material was lysed using lysostaphin, lysozyme, and mutanolysin (Sigma-Aldrich, Zwijndrecht, The Netherlands) as described.⁷ The DNA isolation was finalized with the same kit and robot as described above.

Multiplex PCR design

Using publicly accessible sequences, consensus sequences were created of the regions targeted by published PCR assays aimed at speciation (*gdh*),²⁰ virulence (*epf*),³⁷ and serotyping (*cps1I*, *cps2J*, *cps9H*, *cps7H*).^{24,25} Using conserved regions within these consensus sequences, PCR primer pairs were designed (AlleleID 7.6, PREMIER Biosoft, Palo Alto, CA). The primers were designed to allow amplification of the 6 targets in 1 multiplex reaction (Table 1). Each primer pair was designed for *S. suis*-specific sequences, except the *gdh* primers, which also match the *gdh* of *S. pneumoniae*. This was taken into account by designing an *S. suis*-specific primer for the subsequent TSPE as described below. The *epf* primer pair may also amplify non-functional *epf** variants that contain extra sequences within the target region.²³ This was taken into account when designing the subsequent *epf*-specific TSPE primer (see below). Primers designed for PCR were obtained as unpurified oligonucleotides (Biolegio, Nijmegen, The Netherlands).

Design of primers for TSPE

TSPE primers were designed (PrimerPlex 2.0, PREMIER Biosoft) for amplicon production in multiplex PCR reactions (Table 1). For all TSPE reactions, the primers were designed to hybridize to the sense strand, except for *epf* (see below). To allow use of commercial paramagnetic beads for capture of single-stranded TSPE products, all TSPE primers were designed with specific sequences at their 5'-end (xTAGs) that are compatible with available beads (see below).

For *cps* genes, straightforward design of TSPE primers was possible. For *gdh*, the TSPE primer was designed to explicitly target an *S. suis*-specific single nucleotide polymorphism (3'-end of the oligonucleotide), which in theory enables differentiation between the 2 species *S. suis* and *S. pneumoniae*. This is relevant because the preceding PCR will amplify the selected *gdh* region of both streptococci (see above). For *epf*, a TSPE primer was designed for the anti-sense strand using the following strategy: the primer largely overlaps with the forward PCR primer that runs up to a

Table 1. Oligonucleotides for PCR and target-specific primer extension (TSPE) of *Streptococcus suis* strains.

Target gene	PCR primer sequence (5'–3')	Amplicon size (bp)
<i>cps11</i>	F: GAAAATAATGTTTGGTGCAAGCT R: CGAACTGTTACGAATGACATAATT	180
<i>cps2J</i>	F: TTTGTCGGGAGGGTTACTTG R: CCACTGTTCAAGTGTCAAAAACC	210
<i>cps7H</i>	F: GTGGATTGCGTTCTTGGATTG R: CACCCTGGATAGCCGTAATTG	204
<i>cps9H</i>	F: CAAAGTGTATCAGCAGACAAAAGT R: GTCCCAAATATCATCTTGATCTGAA	216
<i>gdh</i>	F: AAACACATCGGACCTTCACTT R: CCGTAACCAGTTGCTTCTGG	176
<i>epf</i>	F: AGGCGAAAAAGCTATTAACCAG R: ACTGGACTTGTTGAAGCATCA	251
	TSPE primer sequence* (5'–3')	MagPlex-TAG bead
<i>cps11</i>	CTTTATCAAATTCTAATTCTCAAC-TTACATTGTTCAAGACCTCCAA	A073
<i>cps2J</i>	CTATCATTTATCTCTTTCTCAATT-TTTCATTTCTAAGTCTCGCA	A072
<i>cps7H</i>	ACTACTTATTCTCAAACCTCAATA-CCCTGGATAGCCGTAATTGTAC	A033
<i>cps9H</i>	CTTAAACTCTACTTACTTCTAATT-TTCCTTCTGAACCTAAGTTTGT	A056
<i>gdh</i>	TTCAATTCAAATCAAACACATCAT-AGTCAAGACACCTGCATCAAAC	A064
<i>epf</i>	TAACCTTACACTTAACTATCATCTT-GCGAAAAAGCTATTAACCAGTC	A027

* TSPE primers consist of 2 segments (i.e., an xTAG (5'-half) and a complement of the target sequence). The xTAG is complementary to the oligonucleotide on the MagPlex-TAG bead (Luminex). The boundary between the regions is indicated by a hyphen.

known insertion point (between nucleotides 2859 and 2860 of *epf* in *S. suis* strain D282; accession X71881).²³ However, the TSPE primer is 2 nucleotides longer at the 3'-end and reaches over the insertion point. This strategy allows (partial) hybridization of this primer with amplicons from all *epf* variants, but allows only full hybridization and primer extension when targeted amplicons are from an *epf* with no inserts. Primers designed for TSPE were obtained as high-performance liquid chromatography-purified oligonucleotides (Biolegio).

PCR

Conventional PCR assays for serotyping (targeting *cps11*, *cps2J*, *cps7H*, *cps9H*) and detection (*epf*, *gdh*) were performed essentially as described^{20,24,25,37} using a hot-start polymerase (HotStarTaq Mastermix, Qiagen, Venlo, The Netherlands) on a GeneAmp PCR System 9700 (in 9600 run mode; Applied Biosystems, Life Technologies Europe, Bleiswijk, The Netherlands). Multiplex PCRs were performed using a polymerase dedicated to multiplexing (Multiplex PCR Plus Kit, Qiagen; Fig. 1) or an ordinary hot-start polymerase (HotStarTaq Mastermix, Qiagen; all other results) that is less well suited for multiplexing, but is compatible with the DNA used. All PCRs were performed in 25- μ L reactions on a GeneAmp PCR System 9700 (in 9600 run mode): 5 min 95°C, 30 cycles of 30 s at 95°C, 90 s at 50°C, and 90 s at 72°C, followed by 10 min at 68°C. PCR products were analyzed by agarose gel electrophoresis (4% precast E-gel, Invitrogen, Life Technologies Europe) with a molecular

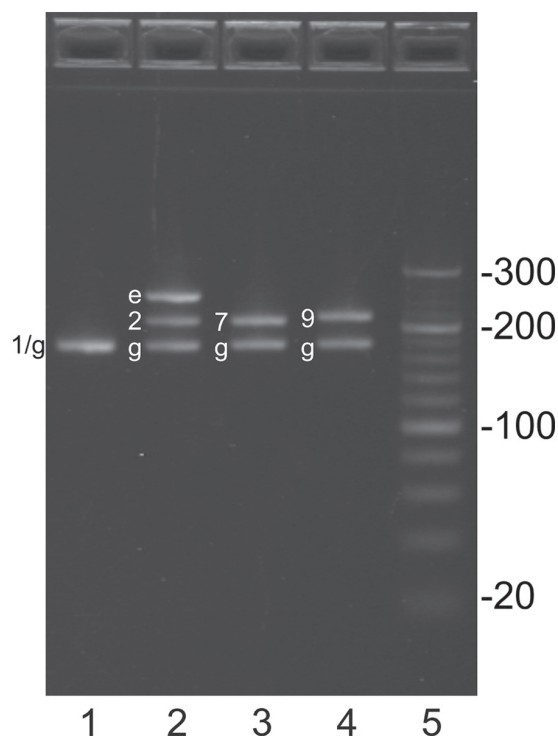


Figure 1. Amplicons generated by multiplex PCR were analyzed on 4% agarose gel. As template, DNA from *Streptococcus suis* strains of serotype 1, 2 (EF+), 7, and 9 (lanes 1–4) was used. Amplicons generated from genes *cps11*, *cps2J*, *cps9H*, *cps7H*, *gdh*, and *epf* are labelled 1, 2, 7, 9, g, and e, respectively. For expected sizes of the various amplicons, see Table 1. The sizes of the 20-bp DNA marker (lane 5) are indicated.

weight marker (O'RangeRuler 20 bp DNA Ladder, Invitrogen, Life Technologies Europe) used in each run.

TSPE step

After multiplex PCR, excess primers and nucleotides were removed using Sepharose (GE Healthcare, Eindhoven, The Netherlands), as described.³⁰ The subsequent TSPE was performed as described for allele-specific primer extension.¹ In short, single-stranded biotinylated products were generated using 5 µL of Sepharose-purified PCR product as template, with 6 TSPE oligonucleotides in 1 reaction mixture containing a special polymerase (Platinum Genotype Tsp DNA Polymerase, Invitrogen) and biotinylated nucleotides (Invitrogen). The run parameters were as follows: 2 min 96°C, 30 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 74°C.

Hybridization

For detection of TSPE products, a mix was prepared containing paramagnetic beads (MagPlex-TAG beads A027, A033, A056, A064, A072, A073, Luminex, 's-Hertogenbosch, The Netherlands) carrying oligonucleotides complementary to the 5'-extensions (xTAGs) of the TSPE primers (Table 1). Hybridization of TSPE products to beads was performed as described.¹ In brief, a 50-µL reaction mixture was prepared in hybridization buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0) containing 5 µL of the TSPE reaction and 1,250 beads of each bead set. In a GeneAmp PCR System 9700 (in 9600 run mode), the mix was denatured (1.5 min at 96°C) and then cooled to allow hybridization of amplicons to beads (37°C for 30 min). The 96-well plates with reaction mixtures were then placed on a magnet (DynaL MPC-96-S, Invitrogen) for 1 min. The supernatant was removed and replaced by 75 µL of streptavidin-phycoerythrin (2 µg/mL; Prozyme, ImmunoSource, Halle-Zoersel, Belgium) in hybridization buffer. The sample was incubated for 15 min at 37°C on a thermal mixer (BioShake iQ shaker, Quantifoil Instruments, Jena, Germany) at 600 rpm.

Analyses of beads were performed at 37°C (Luminex 200 with xPONENT 3.1 software, Luminex) using default settings. For each sample, fluorescence of at least 100 beads per bead set was measured (median fluorescence intensity [MFI]). Where required, cutoffs were calculated for each bead set as the average of the expected negative samples plus 5 times the standard deviation.

Experiments

Evaluation of the multiplex PCR and TSPE was performed using strains of serotype 1, 2 (EF+), 7, and 9. To verify the designed multiplex PCRs, amplicons were generated using a polymerase and buffer combination dedicated to multiplexing, and analyzed by agarose gel electrophoresis. To verify the TSPE designs, amplicons were generated with the

designed multiplex PCR using an ordinary hot-start polymerase, and subsequently used in a TSPE reaction in triplicate. The resulting TSPE products were then analyzed using the suspension array.

Specificity of the suspension array (i.e., the combined procedure of multiplex PCR, TSPE, and hybridization to paramagnetic beads) was evaluated with multiple *S. suis* strains and non-*S. suis* isolates in comparison with conventional PCR assays. These and all other (see below) multiplex PCRs were performed using the ordinary polymerase.

Analytical sensitivities of the suspension array were compared with that of the conventional PCRs using 10-fold serial dilutions of 4 *S. suis* strains. These were tested for the 4 serotype-related *cps* genes. Prior to purification and TSPE, amplicons from the multiplex PCR were also analyzed on agarose gels to check the presence of amplicons as input for TSPE.

Evaluation of the suspension array with field samples was done with 40 tonsillar specimens from pigs originating from a herd with recent *S. suis* infections. Results of the conventional PCRs were used to define samples positive or negative for a particular target, which in turn was used to calculate cutoffs for each bead set of the suspension array (except for *gdh*) and to determine the diagnostic sensitivities and specificities.

Results

Evaluation of the multiplex PCR and TSPE

Gel analysis of amplicons from the multiplex PCR showed that amplicons of expected sizes were generated (Fig. 1; Table 1) with one exception. The *gdh* and *cpsII* amplicons of the serotype 1 strain are of similar size and were not distinguishable as separate products. Analysis of TSPE products showed that, for each target, the suspension array acquired signals that were in concordance with the genes expected to be present in the test strains (Fig. 2; i.e., for all strains a signal was observed on *gdh* beads, whereas for each strain the correct *cps*-specific signal was acquired. As expected, only the serotype 2 (EF+) strain gave a signal for the *epf* beads).

Specificity of the suspension array

Evaluation of the suspension array with multiple *S. suis* strains showed results for *cps* genes and *epf* that matched the results obtained by PCR analysis (Supplementary Table 1), and were in concordance with the expected genetic makeup of the strains. For *gdh*, the suspension array returned substantial signals for the majority of strains, bar serotypes 13, 16, and 17. Further, no significant *gdh* signals were obtained for strains that in retrospect are not *S. suis* (the reference strains for serotypes 20, 22, 26, 32–34). In the conventional PCR analysis for *gdh*, amplicons were generated with all DNA preparations, except for *S. suis* serotype 13.

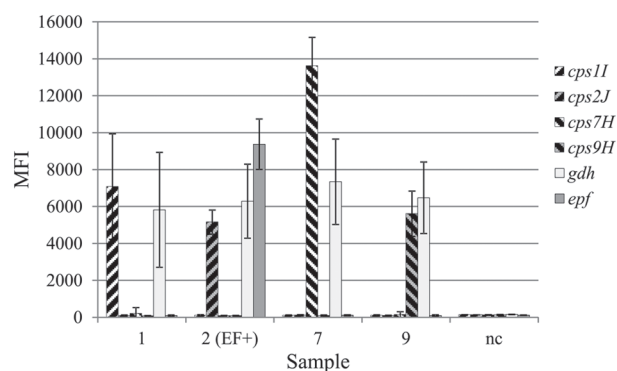


Figure 2. Amplicons generated by multiplex PCR from *Streptococcus suis* strains of serotype 1, 2 (EF+), 7, and 9 were subjected to target-specific primer extension and subsequently analyzed with the suspension array, using a mix of 6 bead types, each carrying specific probes. For each sample, the results for the 6 bead types are indicated; water was used as negative control (nc). The target-specific primer extension and subsequent hybridizations were performed in triplicate; fluorescent signals are given as median fluorescence intensity (MFI) with standard deviations from the 3 experiments.

When the suspension array was evaluated with non-*S. suis* isolates (Supplementary Table 2), on all 6 bead sets the acquired signals remained <300 MFI (i.e., below the calculated cutoffs), except for *S. pneumoniae*, which resulted in a low signal of 341 MFI on the *gdh* beads. As expected, for 2 positive controls (*S. suis* serotype 2 EF+ and EF*), the acquired signals for *cps2J* and *gdh* were above the cutoffs (calculated within the set), as was the signal for *epf* for *S. suis* serotype 2 (EF+), all in concordance with the genetic makeup of these strains.

Analytical sensitivities of the suspension array

For serotypes 1 and 2, the occurrence of positive *gdh* signals in the suspension array matched the presence of amplicons prior to TSPE and hybridization (Supplementary Table 3). For serotypes 7 and 9, the suspension array was more sensitive (one step in the serial dilution) than the multiplex PCRs. Some cross-reactions were observed, such as (low) signals of serotype 9 on the *cps7H* bead, and of serotype 2 on the *cps11* bead. PCRs for *cps* and *epf* genes were more sensitive than the multiplex PCR and suspension array.

Evaluation of the suspension array

Of 40 tested field samples, 10 were positive in the conventional *cps11* PCR; these samples were always positive in 2 or more conventional *cps* PCRs (Supplementary Table 4). More than half (23 of 40) of the samples were PCR-positive for *cps2J*, and, except for sample 11, PCR-positive for *epf* (22 of 23). By conventional PCR, 23 samples were positive for *cps7H*; all of these samples were also PCR-positive for

cps9H, and most samples were also PCR-positive for *cps11* and/or *cps2J*. In fact, most field samples (38 of 40) were PCR-positive for *cps9H*. Although all 40 field samples were PCR-positive for at least 1 *cps* gene, the conventional *gdh* PCR was negative for 5 of 40 samples.

All signals in the suspension array that are designated as positive, were also positive in the corresponding conventional PCR, but for a large number of results from conventional PCR assays, the positive result could not be reproduced by the bead-based assay (Supplementary Table 4). In comparison with conventional PCR, the new assay resulted in low diagnostic sensitivities for *cps* genes (0.30, 0.42, 0.61, 0.61 for *cps11*, *cps2J*, *cps7H*, *cps9H*, respectively) at high diagnostic specificities of 1.00, given that there were no false-positives in the suspension array (Table 2). For *epf*, diagnostic sensitivity of the suspension array was 0.91, also at a diagnostic specificity of 1.00. Diagnostic sensitivity and specificity for *gdh* were not available; a cutoff for the *gdh* bead in the suspension array could not be calculated because true *S. suis*-negative field samples (i.e., PCR-negative for *gdh* and all *cps* genes) were not present in the set tested. Nevertheless, the observed high MFI values on the *gdh* beads suggested that *S. suis* was present in all samples, concordant with the observation that all samples were positive for *gdh* and/or at least one *cps* gene by conventional PCR.

Discussion

Using DNA from *S. suis* reference strains, we confirmed that the proposed assay is sufficiently specific for the *cps* genes, despite the occurrence of some minor cross-reactions. This shows that the new assay can be used to serotype isolates. The design for *epf* was not extensively tested with all possible *epf* variant strains.²³ However, the results show that only a serotype 2 EF+ strain returns a signal, whereas a serotype 2 EF* strain, containing an insert in *epf*, correctly remains negative in the suspension array.

Concerning detection of *gdh*, for several of the reference strains used, there is doubt if these strains are *S. suis*.^{13,18,29} This situation is confirmed by our observation that the disputed *S. suis* serotypes 20, 22, 26, 32, 33, and 34 reference strains are indeed *gdh* negative by the suspension array. Three true *S. suis* strains were missed by the suspension array (the reference strains for serotypes 13, 16, and 17), of which the serotype 13 reference strain was also negative by conventional *gdh* PCR. At the time of design, all available *S. suis* *gdh* sequences matched with the TSPE primer. However, a newly added serotype 16 strain³⁴ indeed has a *gdh* sequence that differs at the TSPE primer binding site, showing that the current design for the *gdh* primers may need revision. The difference in results between the conventional and multiplex PCRs shows that correct speciation using *gdh* depends on the region chosen as target, indicating that unambiguous speciation of *S. suis* is difficult. As an example, a species-specific PCR based on an alternative gene such as *fbpS* recognizes

Table 2. Diagnostic sensitivity (DSe) and specificity (DSp) of the suspension array.

Target gene	Classification of test results for 40 samples generated by 2 assays				Total	DSe	DSp	Cohen kappa
	PCR+SA+	PCR+SA-	PCR-SA+	PCR-SA-				
<i>cpsII</i>	3	7	0	30	40	0.30	1.00	0.39
<i>cps2J</i>	10	13	0	17	40	0.43	1.00	0.40
<i>cps7H</i>	14	9	0	17	40	0.61	1.00	0.57
<i>cps9H</i>	23	15	0	2	40	0.61	1.00	0.13
<i>epf</i>	20	2	0	18	40	0.91	1.00	0.90

PCR = conventional PCR; SA = suspension array; + = positive by PCR or SA; - = negative by PCR or SA. The results of the developed suspension array with field samples (Supplementary Table 4) were used to calculate¹⁶ DSe and DSp with 95% confidence intervals in comparison with the complex of conventional PCR assays. This was done for all target genes except *gdh* (see Supplementary Table 4).

S. suis serotypes 1–34,²⁶ which is in conflict with previous reports on speciation, where for particular serotypes it is disputed if they are *S. suis*.^{13,18,29}

The analytical sensitivity of the conventional PCRs is greater than that of the new multiplex assay. In fact, the conventional PCR is more sensitive than the multiplex PCR part of the assay, even though the same quality of DNA and the same polymerase was used. True multiplexing, with multiple targets present in a sample, was possible with a dedicated PCR mix that is optimized for multiplexing, and resulted in discrete products and similar amounts of amplicons for each targeted gene sequence (Fig. 1). However, the routine procedure used at our laboratory to isolate DNA from tonsillar brush samples, which is based on a commercial DNA isolation kit and the corresponding robot, yielded DNA that was not compatible with a PCR kit dedicated to multiplexing. As a compromise, for all experiments with the suspension array, the amplification was performed with a different, but comparable, PCR kit from the same manufacturer. As this kit is not optimized for multiplexing, this resulted in less efficient and biased DNA amplification, and, as a consequence, reduced sensitivity of the developed multiplex assay. Thus, regarding analytical sensitivity, there is room for improvement by changing the DNA isolation to a method compatible with the use of a polymerase dedicated to multiplexing.

Results with the complex of conventional PCRs showed that, based on the presence of *cps* signals, all 40 tonsil samples tested were *S. suis* positive, and that most samples harbored a mix of *S. suis* strains (Supplementary Table 4). There was a discrepancy with the conventional *gdh* PCR given that only 35 of 40 samples were *gdh* positive, another indication that *S. suis* identification using the *gdh* gene is challenging. For the *gdh* component of the new assay, no diagnostic sensitivities or specificities were calculated because there were no *S. suis*-negative samples. The results of the serotyping by conventional PCRs could not be reproduced by the new assay (Table 2). Nonetheless, in our sample survey, the test correctly identified the presence of *S. suis* serotypes 1, 2, 7, and 9 in a herd from a farm with recent problems related to *S. suis* infections with serotypes 1, 2, and 9. The assay also indicated that strains with serotype 7 were present, a situation not known before our study.

Use of imperfect assays for classification of herds is possible⁵ and has been applied for identification of *Mycobacterium avium*-positive herds with an ELISA that has a low sensitivity.¹⁴ Therefore, even though the sensitivities of the suspension array for *S. suis* are not very high for each separate target gene (Table 2), the assay may be used to screen herds for circulating *S. suis* serotypes. Setting cut-offs for field application is an issue that still needs to be resolved; ideally, a large set of samples would be used, and analyzed with a reference method that can be used to set fixed thresholds.

A 2-step suspension array for serotyping *S. suis* isolates has been described,² wherein the initial multiplex PCR primers are used that carry the sequence required for hybridization, thereby forfeiting the need for a TSPE reaction. This approach will significantly increase the assay speed, but it remains unknown if sensitivity is sufficient for field samples. Our 3-step suspension array is not sensitive enough yet, but a better combination of DNA isolation procedure and multiplex polymerase may improve that, as discussed above. It is expected that with the developed 3-step suspension array, analysis of field samples will require less hands-on time and will be faster than analysis by conventional PCRs and gel electrophoresis, especially when larger numbers are being analyzed.

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Declaration of conflicting interests

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References

- Angeloni S, et al. Allele-specific primer extension (ASPE) SNP typing. In: The xMAP Cookbook. 1st ed. Luminex Corp, 2013:85–92.
- Bai XM, et al. Simultaneous detection of 33 *Streptococcus suis* serotypes using the Luminex xTAG assay. J Microbiol Meth 2015;117:95–99.
- Baig A, et al. Whole genome investigation of a divergent clade of the pathogen *Streptococcus suis*. Front Microbiol 2015;6:1191.
- Braun SD, et al. Fast DNA serotyping and antimicrobial resistance gene determination of *Salmonella enterica* with an oligonucleotide microarray-based assay. PLoS One 2012;7:e46489.
- Christensen J, Gardner IA. Herd-level interpretation of test results for epidemiologic studies of animal diseases. Prev Vet Med 2000;45:83–106.
- Christopher-Hennings J, et al. Opportunities for bead-based multiplex assays in veterinary diagnostic laboratories. J Vet Diagn Invest 2013;25:671–691.
- Cornelissen JBJ, et al. Rapid detection of *Streptococcus uberis* in raw milk by loop-mediated isothermal amplification. J Dairy Sci 2016;99:4270–4281.
- Cunha MV, Inacio J. Overview and challenges of molecular technologies in the veterinary microbiology laboratory. Methods Mol Biol 2015;1247:3–17.
- De Greeff A, et al. Distribution of environmentally regulated genes of *Streptococcus suis* serotype 2 among *S. suis* serotypes and other organisms. J Clin Microbiol 2002;40:3261–3268.
- Dunbar SA. Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. Clin Chim Acta 2006;363:71–82.
- Giles T, et al. Development of a DNA-based microarray for the detection of zoonotic pathogens in rodent species. Mol Cell Probes 2015;29:427–437.
- Goyette-Desjardins G, et al. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent—an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect 2014;3:e45.
- Hill JE, et al. Biochemical analysis, *cpn60* and 16S rDNA sequence data indicate that *Streptococcus suis* serotypes 32 and 34, isolated from pigs, are *Streptococcus orisratti*. Vet Microbiol 2005;107:63–69.
- Hiller A, et al. Prevalence of *Mycobacterium avium* in slaughter pigs based on serological monitoring results and bacteriological validation. Int J Environ Res Public Health 2013;10:4027–4038.
- Lung O, et al. Multiplex PCR and microarray for detection of swine respiratory pathogens. Transbound Emerg Dis 2017;64:834–848.
- Mackinnon A. A spreadsheet for the calculation of comprehensive statistics for the assessment of diagnostic tests and inter-rater agreement. Comput Biol Med 2000;30:127–134.
- Michelet L, et al. High-throughput screening of tick-borne pathogens in Europe. Front Cell Infect Microbiol 2014;4:103.
- Nomoto R, et al. Reappraisal of the taxonomy of *Streptococcus suis* serotypes 20, 22 and 26: *Streptococcus parasuis* sp. nov. Int J Syst Evol Microbiol 2015;65:438–443.
- Okura M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Appl Environ Microbiol 2013;79:2796–2806.
- Okwumabua O, et al. A polymerase chain reaction (PCR) assay specific for *Streptococcus suis* based on the gene encoding the glutamate dehydrogenase. FEMS Microbiol Lett 2003;218:79–84.
- Pappas G. Socio-economic, industrial and cultural parameters of pig-borne infections. Clin Microbiol Infect 2013;19:605–610.
- Silva LM, et al. Virulence-associated gene profiling of *Streptococcus suis* isolates by PCR. Vet Microbiol 2006;115:117–127.
- Smith HE, et al. Repeats in an extracellular protein of weakly pathogenic strains of *Streptococcus suis* type 2 are absent in pathogenic strains. Infect Immun 1993;61:3318–3326.
- Smith HE, et al. Rapid PCR test for *Streptococcus suis* serotype 7. FEMS Microbiol Lett 1999;178:265–270.
- Smith HE, et al. The *cps* genes of *Streptococcus suis* serotypes 1, 2, and 9: development of rapid serotype-specific PCR assays. J Clin Microbiol 1999;37:3146–3152.
- Srinivasan V, et al. Species-specific real-time PCR assay for the detection of *Streptococcus suis* from clinical specimens. Diagn Microbiol Infect Dis 2016;85:131–132.
- Staats JJ, et al. *Streptococcus suis*: past and present. Vet Res Commun 1997;21:381–407.
- Swildens B, et al. Elimination of strains of *Streptococcus suis* serotype 2 from the tonsils of carrier sows by combined medication and vaccination. Vet Rec 2007;160:619–621.
- Tien LHT, et al. Reappraisal of the taxonomy of *Streptococcus suis* serotypes 20, 22, 26, and 33 based on DNA-DNA homology and *sodA* and *recN* phylogenies. Vet Microbiol 2013;162:842–849.
- Van Brunschot SL, et al. A bead-based suspension array for the multiplexed detection of begomoviruses and their whitefly vectors. J Virol Methods 2013;198C:86–94.
- Van Brunschot SL, et al. Development of a multiplexed bead-based suspension array for the detection and discrimination of pospiviroid plant pathogens. PLoS One 2014;9:e84743.
- Vecht U, et al. Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depends on phenotype. Infect Immun 1992;60:550–556.
- Wang K, et al. Development of rapid serotype-specific PCR assays for eight serotypes of *Streptococcus suis*. J Clin Microbiol 2012;50:3329–3334.
- Wang K, et al. Complete genome sequence of *Streptococcus suis* serotype 16 strain TL13. Genome Announc 2013;1:e00394.
- Wertheim HF, et al. *Streptococcus suis*: an emerging human pathogen. Clin Infect Dis 2009;48:617–625.
- Wisselink HJ, et al. Multiplex PCR assays for simultaneous detection of six major serotypes and two virulence-associated phenotypes of *Streptococcus suis* in tonsillar specimens from pigs. J Clin Microbiol 2002;40:2922–2929.
- Wisselink HJ, et al. Detection of virulent strains of *Streptococcus suis* type 2 and highly virulent strains of *Streptococcus suis* type 1 in tonsillar specimens of pigs by PCR. Vet Microbiol 1999;67:143–157.
- Wisselink HJ, et al. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in seven European countries. Vet Microbiol 2000;74:237–248.