Development of a Multiplex PCR Assay for Rapid Molecular Serotyping of *Haemophilus parasuis*

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Haemophilus parasuis causes Glässer's disease and pneumonia in pigs. Indirect hemagglutination (IHA) is typically used to serotype this bacterium, distinguishing 15 serovars with some nontypeable isolates. The capsule loci of the 15 reference strains have been annotated, and significant genetic variation was identified between serovars, with the exception of serovars 5 and 12. A capsule locus and *in silico* serovar were identified for all but two nontypeable isolates in our collection of >200 isolates. Here, we describe the development of a multiplex PCR, based on variation within the capsule loci of the 15 serovars of H. parasuis, for rapid molecular serotyping. The multiplex PCR (mPCR) distinguished between all previously described serovars except 5 and 12, which were detected by the same pair of primers. The detection limit of the mPCR was 4.29×10^5 ng/µl bacterial genomic DNA, and high specificity was indicated by the absence of reactivity against closely related commensal *Pasteurellaceae* and other bacterial pathogens of pigs. A subset of 150 isolates from a previously sequenced H. parasuis collection was used to validate the mPCR with 100% accuracy compared to the *in silico* results. In addition, the two *in silico*-nontypeable isolates were typeable using the mPCR. A further 84 isolates were analyzed by mPCR and compared to the IHA serotyping results with 90% concordance (excluding those that were nontypeable by IHA). The mPCR was faster, more sensitive, and more specific than IHA, enabling the differentiation of 14 of the 15 serovars of H. parasuis.

*H*aemophilus parasuis is a Gram-negative bacterium commonly found in the upper respiratory tract of the pig, and it was identified in 1910 as the causative agent of a globally prevalent systemic disease of pigs known as Glässer's disease. The more severe presentations of this disease include arthritis, meningitis, polyserositis, septicemia, and pneumonia (1–5). Based on statistics from the United States, *H. parasuis* is the leading cause of mortality (alongside the porcine reproductive and respiratory syndrome [PRRS] virus) in nursery herds, and it is the third most important bacterial pathogen affecting finisher herds (6). *H. parasuis* also contributes to a multifactorial porcine respiratory disease complex, the leading cause of mortality in grower-finisher pigs in the United States (7). Diagnostic submissions to veterinary investigation centers of the Animal and Plant Health Agency (APHA) in 2013 and 2014 recorded the highest annual rates of diagnosis of disease incidents due to *H. parasuis* in England and Wales since 2002 (8, 9). In the third quarter of 2013, the diagnostic rate reached nearly 8% of diagnosable submissions (8, 9). This disease characteristically manifests postweaning and is associated with the loss of maternally derived antibodies and the endemic presence of the bacterium in herds (1, 5).

Treatment and prevention of Glässer's disease are implemented via strategic delivery of penicillin-based antimicrobials in feed or water. Ongoing treatment may be administered to successive batches of susceptible pigs for several months after an outbreak to ensure the full recovery of the herd (5, 10, 11). Regular medication of farmed livestock is of concern, as antimicrobial resistance may be selected by the prolonged use of these drugs.

Antimicrobial resistance in *H. parasuis* has been reported in China and Spain, where the majority of *H. parasuis* strains are resistant to enrofloxacin and trimethoprim (10, 12, 13). Control of stock movement in and out of the herd is currently the best method of prevention, as it reduces the risk of introducing new strains (5, 14, 15).

The current commercially available vaccines are bacterins, which are protective only against strains of the same serovar (16–18), and which primarily target the disease-causing serovars 4 and 5, with limited cross-protection against others (5, 19, 20). It is possible to make autogenous vaccines in response to an outbreak of Glässer's disease, which can be useful if the serovar is different

from that targeted by the commercial vaccines (21), but this is an expensive and time-consuming option. In addition, multiple isolates, often of different serovars, may be present within an individual or a herd, which can result in the wrong isolate being chosen for the production of the autogenous vaccine.

Serotyping is the most frequently used subtyping method for *H. parasuis*, as it is important for guiding the vaccination strategy to try to prevent future outbreaks. The current serotyping scheme, based on reactions between antisera and surface antigens, classifies the bacteria into 15 serovars, with a considerable number of nontypeable (NT) isolates observed (22, 23). Most commonly isolated from the field are serovars 4, 5, and 13 (24–27). However, isolates are collected predominantly from severely affected individuals or clinical cases from within a herd, with only a single colony studied from those cultured from a swab. As multiple infections of the same individual and within herds can occur (25, 27–29), there may be additional isolates that contribute to disease in animals that are not commonly investigated. The site of isolation is also very important, as isolates cultured from the joints or from meninges have survived serum killing and phagocytosis and so are highly likely to be virulent; however, if samples are taken from the upper respiratory tract or the lung, there is a higher chance of the isolate being part of a coinfection rather than the isolate responsible for the disease. This might well introduce sampling bias, and the relative proportions of different serovars among isolates actively causing disease and carriage isolates in pig populations may vary from the commonly reported serovars 4, 5, and 13 (24–27).

The Kielstein-Rapp-Gabrielson serotyping scheme was the first to identify the 15 serovars of *H. parasuis* in 1992 using the gel immunodiffusion assay (GID) (23), which has since been superseded by an indirect hemagglutination assay (IHA) (30–32); this has increased the proportion of typeable strains from 60% to 80%. An isolate may be reported as nontypeable if there is no observable reaction, or when four or more different antisera react with the same isolate. A serotyping result can include cross-reactions when two or three antisera react with an isolate, and this is common for field isolates using both serotyping methods (23, 25, 30, 33). In these circumstances, the strongest agglutination reaction is chosen as the main serotyping result, but this can be dependent on visual interpretation by the worker, so human error is introduced into the test. Therefore, even with 80% of isolates being typeable, this success rate is susceptible to errors that reduce accuracy. Improvements in the accuracy of *H. parasuis* serotyping would aid the understanding of the epidemiology of this pathogen and allow optimization of vaccination strategies for the prevention of disease.

There are other drawbacks of the IHA serotyping assay, including the difficulty of consistently producing specific antisera against several reference strains (30), variation in growth conditions or growth rates between isolates, the very small number of laboratories that currently perform this test, and the repeatability or robustness of methods and results between these laboratories (23, 31, 34, 35). The method is also time-consuming, expensive, and requires pure culture of an isolate.

Molecular typing should be considered a potentially more accurate and consistent test. These techniques have been developed for other bacteria based on the genes involved in biosynthesis of extracellular polysaccharide structures, such as lipopolysaccharides (LPS) or capsules (36–39). These are also likely to be the dominant components of the serotyping antigens for *H. parasuis* based on the antigen preparation techniques for both the GID and IHA methods (22, 23, 30, 31). Genes encoding these surface components were therefore the elements of the genome investigated for molecular serotyping markers. An analysis of the first complete *H. parasuis* genome sequence (strain SH0165) identified a 14-kb polysaccharide biosynthesis region that was proposed to encode O antigen, with 12 coding sequences in the same transcriptional direction. It was later proposed that this is in fact a group 1 capsule locus based on the presence of the homologues of the *wza*, *wzb*, and *wzc* genes, and it is responsible for considerable serovar-specific variation (40–43). Furthermore, there is a strong association between the presence of particular capsule loci and serotyping results (44), with 85% of the reference strains studied having the same serotyping result from *in silico* analysis and IHA. Those isolates with different results matched to one of the cross-reactions in the IHA result. In addition, isolates that had been nontypeable (NT) by IHA contained a capsule locus that matched one of the 15 reference strains, with two exceptions (43, 44). These two isolates had capsule genes similar to those identified in serovars 6 and 8, but they had not been assembled onto a single contiguous sequence (contig) or capsule locus and so further investigation.

Here, we describe the design of a molecular serotyping PCR, based on variations within the capsule loci, capable of discriminating between 14 of the 15 serovars of *H. parasuis*. In addition, a new species-specific molecular marker for *H. parasuis* was identified and included in the multiplex PCR (mPCR).

MATERIALS AND METHODS

Isolate collections. For the design of this molecular serotyping test, we used a previously described (44) collection of 212 isolates of *H. parasuis*, 117 of which had been serotyped by IHA. This collection included isolates cultured from pig tissues during diagnostic investigations at the Animal and Plant Health Agency (APHA) from farms in England and Wales between 1993 and 2011, isolates from Denmark, Spain, and Australia, and the 15 serotyping reference strains. This collection included disease- and nondisease-associated isolates, all of which had their genomes sequenced by genomic DNA (gDNA) extraction and paired-end Illumina sequencing, as described previously (44). The genome sequences of these isolates were examined for the presence of a capsule locus, and for all but two of them, a serovar was predicted *in silico* based on the capsule genes (44). A subset of 150 isolates from this original collection was used for the validation of the mPCR, 117 of which had been serotyped by IHA and for all of which a serovar had been predicted by *in silico* analysis. This subset included isolates representing all 15 serovars and those previously serotyped, including those with cross-reactions (*n* = 22), all nontypeable isolates (*n* = 19), and a selection of isolates that had not been serotyped (*n* = 33).

An additional 84 disease-associated isolates of *H. parasuis* were collected by the APHA during 2013 and 2014; we called this the additional isolate collection. Sixty-six of these isolates were serotyped by Innovative Veterinary Diagnostics (IVD), Germany, using IHA, and were of a variety of serovars (serovars 1, 2, 4, 5, 6, 9, 13, 14, and 15), in addition to nontypeable isolates (*n* = 15). Nine of the isolates had cross-reactions reported in their serotyping results. The remaining 18 isolates had not been tested by IHA and so were of unknown serovar. This additional isolate collection with unknown capsule loci did not contribute to the original design of serovar-specific markers and therefore enabled an objective evaluation of the new mPCR.

Isolates of closely related *Pasteurellaceae*, including *Actinobacillus indolicus*, *Actinobacillus minor*, *Actinobacillus porcinus*, and *Actinobacillus porcitonsillarum*, were identified from routine diagnostic investigations at the APHA and also had their genomes sequenced (European Nucleotide

TABLE 1 Serotyping multiplex primers and estimated product sizes

Gene	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Serovar target	Product size (bp)
funB	CTGTGTATAATCTATCCCCGATCATCAGC	GTCCAACAGAATTTGGACCAATTCCTG	1	180
wzx	CTAACAAGTTAGGTATGGAGGGTTTTGGTG	GGCACTGAATAAGGGATAATTGTACTG	2	295
glyC	CATGGTGTTTATCCTGACTTGGCTGT	TCCACATGAGGCCGCTTCTAATATACT	3	650
wciP	GGTTAAGAGGTAGAGCTAAGAATAGAGG	CTTTCCACAACAGCTCTAGAAACC	4	320
wcwK	CCACTGGATAGAGAGTGGCAGG	CCATACATCTGAATTCCTAAGC	5 or 12	450
gltI	GATTCTGATGATTTTTGGCTGACGGAACG	CCTATTCTGTCTATAAGCATAGACAGGAC	6	360
funQ	CTCCGATTTCATCTTTTCTATGTGG	CGATAAACCATAACAATTCCTGGCAC	7	490
scdA	GGAAGGGGATTACTACTACCTGAAAG	CTCCATAGAACCTGCTGCTTGAG	8	650
funV	AGCCACATCAATTTTAGCCTCATCA	CCTTAAATAGCCTATGTCTGTACC	9	710
funX	GGTGACATTTATGGGCGAGTAAGTC	GCACTGTCATCAATAACAATCTTAAGACG	10	790
amtA	CCATCTCTTTAACTAATGGGACTG	GGACGCCAAGGAGTATTATCAAATG	11	890
gltP	GCTGGAGGAGTTGAAAGAGTTGTTAC	CAATCAAATGAAACAACAGGAAGC	13	840
funAB	GCTGGTTATGACTATTTCTTTCGCG	GCTCCCAAGATTAAACCACAAGCAAG	14	730
funI	CAAGTTCGGATTGGGAGCATATATC	CCTATATCATTTGTTGGATGTACG	15	550
HPS_219690793	ACAACCTGCAAGTACTTATCGGGAT	TAGCCTCCTGTCTGATATTCCCACG	All	275

Archive accession no. ERS132116, ERS132148, ERS132149, ERS132152 to ERS132156, ERS132158 to ERS132160, ERS132162 to ERS132165, ERS132169, and ERS132170). These genomes were evaluated using BLASTn against the primers designed for *H. parasuis*, as they are from species that are most likely to cross-react. These isolates together with further field isolates of *Actinobacillus pleuropneumoniae* ($n = 3$), *Bordetella bronchiseptica* ($n = 1$), and *Streptococcus suis* ($n = 3$), all common bacteria in the upper respiratory tracts of pigs, were used in this study as part of a negative-control panel for the mPCR.

Species-specific marker design. A Perl script was used to produce a draft core genome of *H. parasuis* using the genome sequences of the original collection ($n = 212$) (44). The protein-coding sequences from the published complete genome of *H. parasuis* SH0165 (45) were compared to the *H. parasuis* genomes using tBLASTn. If a coding sequence had 80% identity over 80% of the length of the gene to a contig in all of the *H. parasuis* genomes, the gene was considered to be part of the core genome. This list represents the most conserved genes in the *H. parasuis* genome. The SH0165 Fasta sequence for each gene from the core genome was compared to the nonredundant (nr) NCBI database using BLASTn to identify genes with matches to *H. parasuis* only. These core genes were also compared by BLASTn with the genome sequences of the other *Pasteurellaceae* isolates. Genes with matches in *H. parasuis* only were taken forward as potential species-specific markers. The alignments of the genes were assessed using alistat (46) to study the average alignment identity and minimum alignment identity between two sequences in the alignment. Genes with >95% average alignment identity were chosen to create a short list of potential species-specific markers with a variety of amplicon sizes.

Serotyping mPCR design. The capsule loci of the 15 serovar reference strains were previously sequenced and annotated (43). The majority of isolates of the same serovar shared high levels of identity in their capsule loci, as expected (>95% for the majority of serovars) (44). Where loci did not match within the same serovar, the majority of the capsule loci matched to a recorded cross-reaction (44). Twenty-two isolates in the original isolate collection had recorded cross-reactions, and four of these isolates matched to the minor reaction rather than the dominant serovar in the cross-reaction results. A further 10 isolates in the original isolate collection had results that were different from the IHA serotyping result. The agreement between the IHA serotyping and the capsule loci was tested using an unweighted Cohen's kappa test, excluding the NT isolates (47). Differences in the gene composition were found between the capsule loci of the reference strains, i.e., between different serovars, with the exception of serovars 5 and 12, which have 97% identity across the capsule locus (37); a short list of genes found in only one or two of the 14 known capsule

loci was made, from which primers were designed to distinguish between the serovars. The original capsule variation diagram from Howell et al. (43) has been adapted to show the gene differences and target genes for the multiplex design (see Fig. S1 in the supplemental material).

Primer design. Primer design for the species-specific marker and serotyping markers was as follows. Primer3 (http://www.bioinformatics.nl /cgi-bin/primer3plus/primer3plus.cgi/) was used to design primers between 21 and 30 bases in length, with 40 to 60% G+C content, based on recommendations for multiplex PCR (mPCR) design. The primers for each gene were compared, using BLASTn with a word size of 7, to the nr database and the closely related *Pasteurellaceae* bacterial genomes to check for any nonspecific primer matches that would rule out any primer sequences. The primers were also compared to the *H. parasuis* genomes using BLASTn to look for those that matched all of the expected isolates with only one match and 100% identity. All those that passed these checks were then aligned against the target gene, and product sizes were estimated based on all combinations of primers. For several genes, the primers had to be redesigned manually when no suitable primer met the requirement for the range of product sizes. Primer-dimer and hairpin structures were predicted for all of the primer combinations using National Institute of Standards and Technology Primer Tools (http://yellow .nist.gov:8444/dnaAnalysis/primerToolsPage.do), and any problematic primers were removed from the short list. A pair of primers was chosen for each gene, which would give approximately 20 to 50 bp separation between all amplicons when combined into an mPCR. Primers were obtained in dehydrated desalted form from Sigma-Aldrich (Haverhill, Cambridge, United Kingdom). The final target genes and primers for the mPCR are shown in Table 1.

Primer optimization. All primers were initially tested using gradient PCRs using One*Taq* Quick-Load 2× master mix with standard buffer (New England BioLabs), according to the product specifications and protocols. Amplification of the targets was initiated at 94°C for 30 s, followed by 30 cycles of 3-step cycling comprising denaturation at 94°C for 30 s and annealing at a temperature range for the gradient PCR between 52 and 64°C for 30 s, 68°C for 60 s for extension, and a final extension of 68°C for 5 min. Each PCR mixture contained 12.5 μl of One*Taq* Quick-Load 2× master mix, 0.25 μl of each primer (at 20 μM each), 2 μl of gDNA for each isolate (at >10 ng/μl), and 10 μl of UltraPure H$_2$O (Life Technologies) to a final volume of 25 μl. For the individual serovar-specific primers, the reference strain of each serovar was used as a positive control, and an isolate from a different serovar was selected as a negative control. Ultra-Pure H$_2$O was used as a negative control for all PCRs. Gel electrophoresis was performed in a 2.0% agarose gel in 1× Tris-borate-EDTA (TBE) with 5% SYBR Safe dye (Invitrogen) at 120 V for 50 min using the Quick-Load
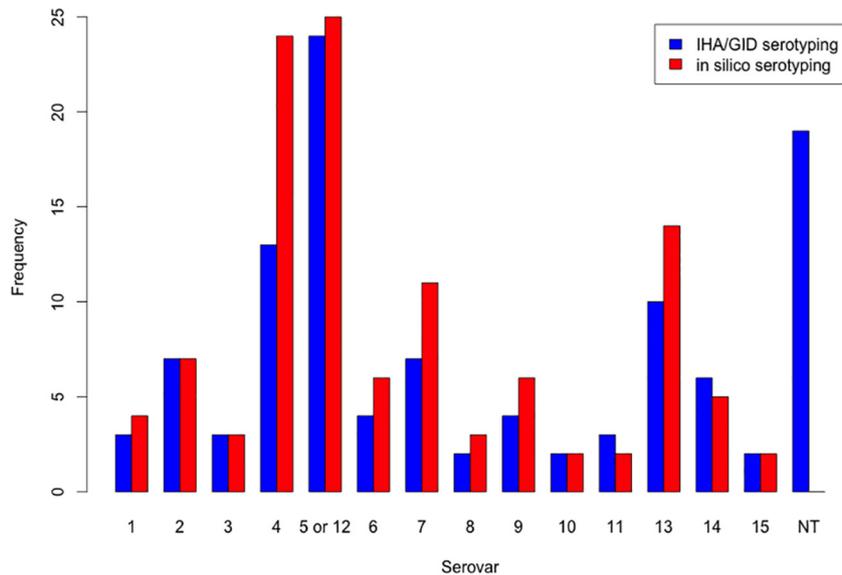
FIG 1 Serovar prevalence of 117 isolates that had been serotyped by IHA (blue) in comparison with the *in silico* serovar predicted for the same isolates based on analysis of the capsule loci (red). The agreement between the IHA and *in silico* serovar prediction was tested using an unweighted Cohen's kappa test, with a *P* value of 0.0000128, excluding the NT isolates.

100-bp DNA ladder (New England BioLabs) as the molecular size standard. All results were analyzed using a GelDoc XR imager (Bio-Rad). Each pair of primers was then tested on the panel of reference strains for the 15 serovars (see Table S1 in the supplemental material) using 25-µl PCRs and the PCR protocol described above, with a consensus annealing temperature of 64°C from the individual gradient PCRs. PCR purification was performed using a 50-µl PCR and the QIAquick PCR purification kit (Qiagen), as per the manufacturer's instructions. PCR products were sequenced using the Source BioScience Sanger sequencing service. The sequences of the products were aligned with the target gene and primers using SeaView (48).

One-step mPCR. The successful primers for the mPCR were combined to create a 50 µM primer mix using 1× Tris-EDTA (TE) buffer. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to the PCRs at 1% of the total reaction volume, and the annealing temperature was reduced to 58°C to improve the production of bands of equal intensity. The primer mixes were also optimized, aiming for equal intensity for each amplicon, with a ratio of 1:0.25 serovar-specific primers to species-specific primers. The final PCR mix included 12.5 µl of One*Taq* Quick-Load 2× master mix, 3 µl of the primer mix, 2 µl of gDNA for each isolate (at >10 ng/µl), and 7.25 µl of UltraPure H_2O to a final volume of 25 µl. Gel electrophoresis for the mPCR was extended to 90 min for better separation of the amplicons.

The mPCR was tested on 234 isolates (a subset of 150 isolates from the original isolate collection and the additional isolate collection of 84 isolates) and was repeated in triplicate using separate master mixes to demonstrate the repeatability and accuracy of the mPCR. For the subset of the original isolate collection, the mPCR results were compared to the *in silico* serovar predictions. For the additional isolate collection, it was only possible to compare the mPCR result to the IHA serotyping result if known.

Genomic DNA extraction requires pure culture, can be time-consuming, and adds additional cost to the diagnosis procedure; therefore, colony PCR methods can be more convenient for diagnostic laboratories. Therefore, for comparison, 20 isolates from the additional isolate collection were also tested using a colony PCR method. A loopful of bacteria from a passaged plate of pure culture was resuspended in 50 µl of UltraPure H_2O, which was heated to 100°C for 30 min and centrifuged at 4,000 × *g* for 1 min, and the supernatant was used in the mPCR reaction. The same volume of supernatant was used in the mPCR reaction mixture as the volume that was used for pure genomic DNA. The results of mPCR were compared to those of the IHA serotyping method (where available).

Limit of detection of the mPCR. The concentration of gDNA was measured for five reference strains (strain name-serovar: HS145-S1, SW140-S2, Nagasaki-S5, C5-S8, D74-Aus–S9, and IA84/17975-S13) using a Qubit fluorometer (Life Technologies) with broad-range standards. Six serial dilutions of this DNA in UltraPure H_2O were used as the template in the mPCR to estimate its limit of detection. This was then calculated in genomes per microliter based on the average genome size of 2.26 Mb.

RESULTS

Design of the serotyping mPCR. Based on the *in silico* analyses of the capsule loci (44), a serovar was predicted for all except two of the *H. parasuis* isolates in the original collection (*n* = 212), including those that were previously determined to be NT using the IHA method (44). Cohen's kappa (47) was used to test the agreement between the IHA and *in silico* analyses (for isolates for which both results were available) and was statistically significant (*P* < 0.01), with the individual serovars treated as categories. The two exceptions had incomplete capsule locus sequences, but the genes identified were highly similar to those from capsule loci from serovars 6 and 8.

Fourteen isolates were discrepant between the *in silico* serovar prediction and the IHA results, with four isolates matching to the cross-reaction: a serovar 12 with cross-reactions to serovars 2 and 4 result was identified as a serovar 2 isolate, and serovar 7 isolates with cross-reactions identified as serovar 4. The remaining isolates were a serovar 2 identified as a serovar 1, a serovar 7 identified as a serovar 9, a serovar 7 with serovar 2 cross-reaction identified as a serovar 4, three serovar 7 isolates identified as serovar 4, a serovar 11 identified as a serovar 13, a serovar 13 identified as a serovar 5 or 12, and two serovar 14 isolates identified as a serovar 13.

Given the success of predicting serovar by *in silico* analysis, we assessed the serovar prevalence of the 117 isolates, which had been serotyped by IHA and were compared with the *in silico* prediction of the serovar of those isolates in the original collection (Fig. 1)
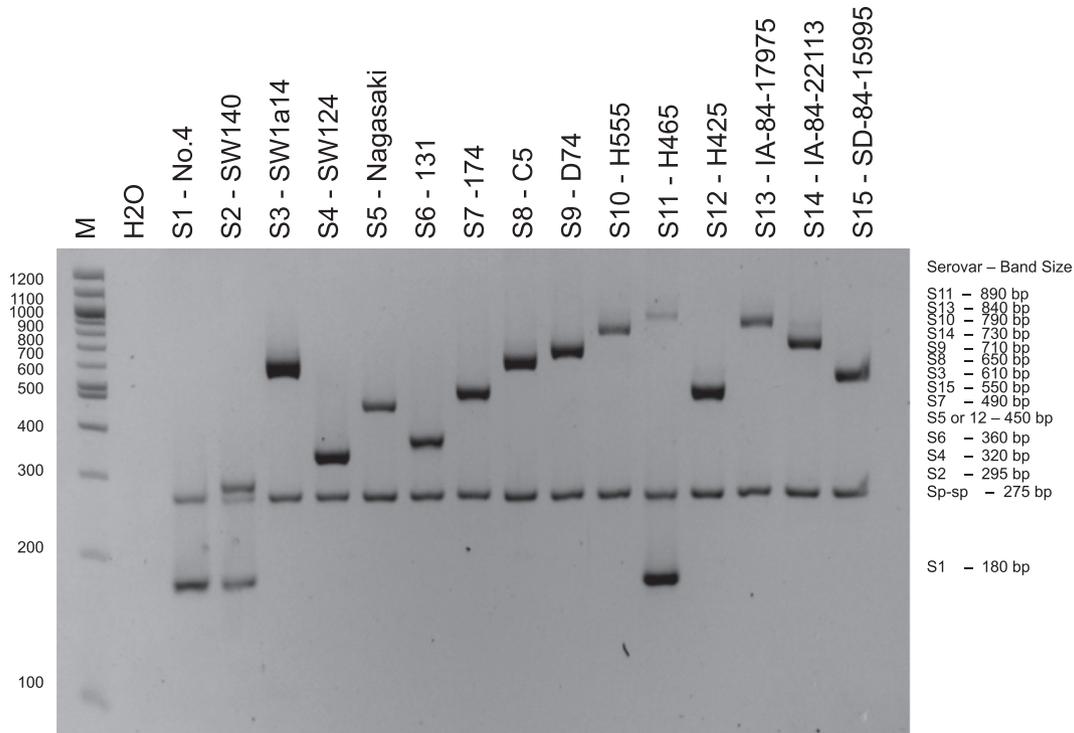
FIG 2 Band patterns for the molecular serotyping PCR for all 15 serovars of *H. parasuis*. Lane M, Quick-Load 100-bp DNA ladder (NEB); S1 to S15 represent the 15 serovars of *H. parasuis*. Sp-sp, species-specific marker.

(44). From the original IHA serotyping results, serovars 5, NT, 4, 7, and 13 were the most prevalent serovars, in order of descending frequency. In comparison, the *in silico* results for all isolates showed that serovar 5 was the most prevalent, closely followed by serovar 4 and then by serovars 13 and 7, and none of the isolates were NT by the *in silico* analysis, in comparison to 19 out of 117 that were NT by IHA serotyping.

The large amount of genetic variation between the capsule loci of the 15 serovars was chosen as the target for a molecular serotyping assay. The assay was designed using a wide variety of genes from within the capsule loci, including an aminotransferase, glycosyltransferases, O-antigen flippase, and genes with unknown function; overall, these genes share <51% identity at the nucleotide level. The target genes among the variable region can be seen in Fig. S1 in the supplemental material. It was not possible to detect differences between serovars 5 and 12 based on the DNA sequences of the capsule loci from any of the examples in the original isolate collection. Even upon detailed analysis of the whole genomes of the serovar 5 and 12 isolates, it was not possible to identify sequence markers to distinguish between these serovars, indicating that there must be a subtle difference in expression of a gene or genes, or that the difference between serovars 5 and 12 is an artifact of the IHA typing antibodies. This finding is consistent with the high frequency of cross-reactions between these serovars according to the IHA test (30). At least one target gene per serovar was identified (counting serovars 5 and 12 as the same serovar), except for serovar 1, for which the same gene was also identified in serovars 2 and 11. A gene of unknown function (*funB*) was chosen as the marker for serovar 1, which was also identified in serovar 11 and was highly similar to another gene of unknown function in serovar 2 (*funE*). The distinguishing prim-
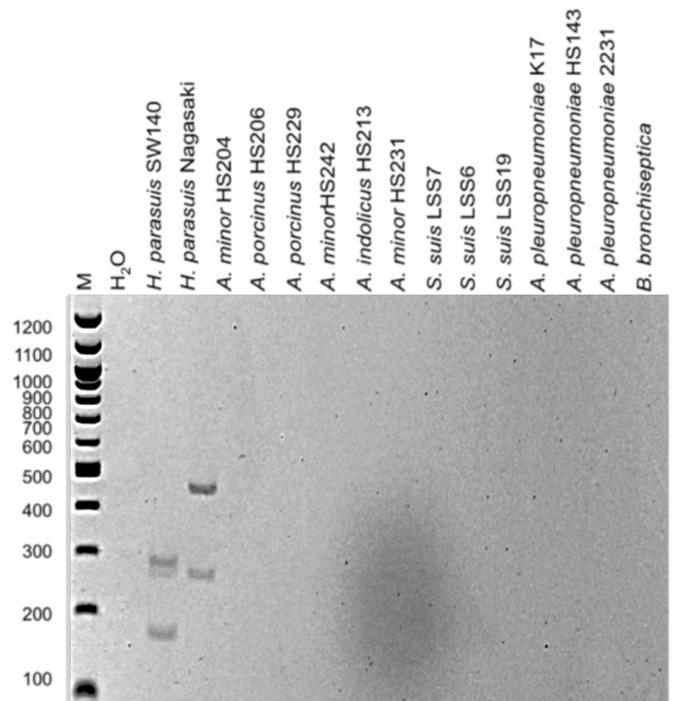

FIG 3 Negative-control panel showing specificity of the primer sets across a range commensal and pathogenic bacteria found in the pig respiratory tract (*H. parasuis*-positive controls serovar 2 and 5, *A. minor*, *A. porcinus*, *A. indolicus*, *S. suis*, *A. pleuropneumoniae*, and *B. bronchiseptica*). Lane M, Quick-Load 100-bp marker (NEB) and H_2O as the negative control.
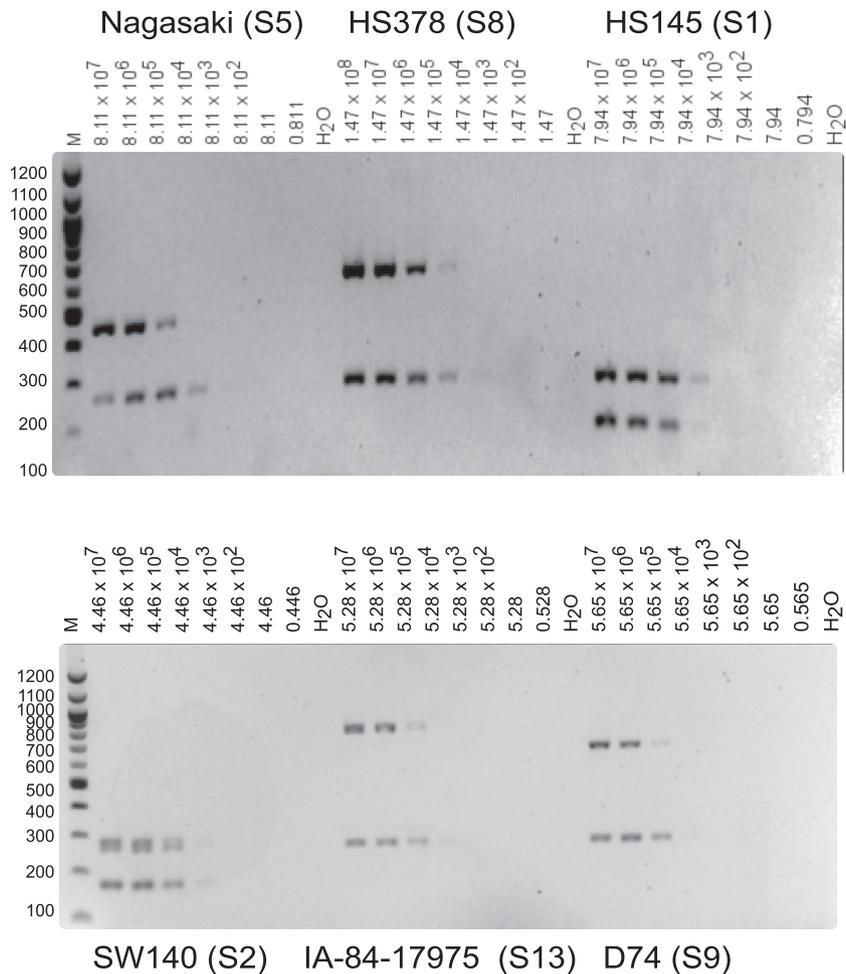
FIG 4 Determination of the limit of detection for the serotyping multiplex based on pure genomic DNA for the reference strains of serovars 1, 2, 5, 8, 9, and 13. The unit of genomes per microliter is used. Lane M, Quick-Load 100-bp marker (NEB) and H_2O as the negative control.

ers for serovar 2 were designed against a divergent *wzx* gene, and the *amtA* gene was used to identify serovar 11 (Fig. 2). By individually testing the serovar-specific primer pairs, it was shown that each pair gave an amplicon of the expected size, each of which was produced only by the expected serovar (Fig. 2) and each of which had the correct DNA sequence. The banding patterns produced by the reference strains in the serovar-specific PCRs, and the new *H. parasuis* species-specific marker, with an amplicon size of 275 bp, is shown in Fig. 2. This species-specific marker (HPS_219690793, with unknown function) was chosen from a short list of highly conserved genes from the core genome, as it fit best with the serovar-specific amplicon sizes.

During the optimization of the mPCR, PCR product purification was performed for each pair of primers using two or three isolates of each serovar, and alignments of the sequenced PCR products with the target gene showed that they were the correct products. The specificities of the serotyping mPCR primers were tested against six other species commonly found in the upper respiratory tract of the pig, including closely related *Pasteurellaceae* and other pig pathogens. No products were amplified from these other species, strongly indicating that these primers are specific for *H. parasuis* (Fig. 3). Using DNA isolated from six reference strains of *H. parasuis*, the average minimum concentration of DNA de-

tectable by the mPCR was determined to be 1 ng/μl for an individual pure gDNA preparation or 4.29×10^5 genomes/μl across the serovars (Fig. 4).

Validation of the serotyping multiplex. The molecular serotyping assay was validated using 150 isolates covering all 15 serovars, including 117 isolates that were previously serotyped by IHA, 19 of which were deemed nontypeable. A summary of the results of this validation exercise and a comparison with the original IHA results and *in silico* serovars are shown in Table 2. The mPCR produced the predicted amplicons at the expected sizes based on the predicted serovar from the *in silico* analyses (44) and therefore was 100% accurate. A serovar was assigned to every isolate by the mPCR, and no cross-reactions were observed. The two isolates with incomplete capsule loci were typed as serovars 6 and 8 by the mPCR. For 33 isolates, the serovar identified by IHA was different from that assigned by the *in silico* or mPCR method. These included the 19 isolates that were NT by IHA, which were identified as serovars 4, 5, 6, 7, 8, 9, 13, and 14 by the mPCR (Table 2), and the remaining isolates matched to the minor cross-reactions or the previously mentioned discrepancies between the capsule types and the IHA results.

A summary of the results of the mPCR for the additional collection of 84 isolates, collected during 2013 and 2014, is shown in

TABLE 2 Summary of the mPCR serotyping results from 150 isolates, showing that the majority of isolates tested had the same result by IHA serotyping as by the mPCR (n = 84)[a]

Serovar by mPCR	Serovar by IHA																Total no. of isolates
	1	2	3	4	5 or 12	6	7	8	9	10	11	13	14	15	NT	Unknown	
1	3	1														2	6
2		6			1											4	11
3			2													0	2
4				13			7								4	5	29
5 or 12					25							1			1	5	32
6						4									2	4	10
7							4								7	4	15
8								2							1	2	5
9							1		4						1	1	7
10										2						0	2
11											2					0	2
13												1	9	2	2	3	17
14													6	1		1	8
15														2		2	4
NT																0	0

[a] All nontypeable (NT) isolates were assigned a serovar by the mPCR (n = 19). An additional subset of 40 isolates was tested with the mPCR that had not been tested by IHA serotyping (unknown), all of which were assigned a serovar using the mPCR.

Table 3. Of the 66 isolates previously tested by IHA, a serovar was determined only in 51, and 15 were classed as NT, all of which were assigned a serovar by the mPCR. There were also six isolates in this collection that were assigned different serovars when tested by mPCR versus IHA. Overall, the IHA serotyping and the *in silico* serovar predictions were 90% concordant. From these results, it is clear that serovar 4 was the most prevalent disease-causing serovar in the United Kingdom in the period of 2013 to 2014, with serovar 5 being the next most prevalent. All results from the colony PCR were identical to the results using gDNA (data not shown).

DISCUSSION

We have developed a multiplex PCR for rapid molecular serotyping of *H. parasuis* based on genetic variation within its capsule locus. This mPCR discriminated between all serovars of *H. parasuis* except serovars 5 and 12, in which the capsule loci are identical (43). The high similarity in gene content of the capsule loci of serovars 1, 2, and 11, which is likely to be due to diversification from a single precursor capsule locus (43), made the identification of a single specific marker for these serovars more difficult; however, we have shown that they can be reliably distinguished using the primer pairs described here.

So far, despite our extensive efforts, no gene to differentiate between serovars 5 and 12 has been identified from the available whole-genome sequences of these serovars. In the future, it may be possible to identify a definitive genetic determinant that is responsible for the separation of these two serovars, but it is also possible that these are in fact not separate serovars. To determine if serovars 5 and 12 really are distinct, it may be necessary to study more closely their capsule structures or the composition of the antigens used in the IHA serotyping assay. This might point, for example, to a difference in gene expression rather than the presence or

TABLE 3 Summary of the mPCR serotyping results of the additional isolate collection of United Kingdom isolates from 2013 and 2014 (n = 84)[a]

Serovar by mPCR	Serovar by IHA																Total no. of isolates
	1	2	3	4	5 or 12	6	7	8	9	10	11	13	14	15	NT	Unknown	
1	4																4
2		3														2	5
3																	0
4				17											2	6	25
5 or 12					11										4	4	19
6						1										1	2
7				1											3	3	7
8						1											1
9									1								1
10																1	1
11																	0
13									2		2	1			1	1	7
14				1									4				5
15															2	5	7

[a] Cross-reactions in the IHA result were ignored for this comparison. All nontypeable (NT) isolates were assigned a serovar by the mPCR. The unknown isolates had not been serotyped by IHA at the time of testing with the mPCR, but all were assigned a serovar using this mPCR.

absence of an allele, as the determinant of the difference between serovars 5 and 12 increased by typing antisera. In the United Kingdom, the Porcilis Glässer vaccine cross-protects (18) between serovars 5 and 12, and so no immediate negative consequences can be seen from the grouping of serovars 5 and 12 in this mPCR assay.

Conventionally, IHA serotyping is considered the gold standard with which to compare our mPCR results. However, IHA serotyping has several well-known drawbacks (nontypeable isolates, cross-reactions, and difficulties in producing antisera) that make it somewhat unreliable and difficult to perform. We previously identified a high level of association between the capsule loci or capsule type of an isolate and the IHA serotyping results (44). Based on these results, we have proposed that the capsule locus is likely to encode the dominant component of the serotyping antigens (43, 44). All nontypeable isolates tested with the mPCR were assigned to a capsule type, with only 12% of isolates assigned to a different serovar than that predicted by IHA excluding NT isolates. The majority of the isolates with cross-reactions matched to the strongest cross-reaction, but four isolates matched to the minor cross-reaction in the serotyping result. The accuracy of the mPCR can be considered in two ways. First, if we compare the mPCR results to the IHA results, taking the IHA results as the gold standard, the mPCR was 87% accurate for isolates of known serovar for the original collection and 78% accurate for the additional isolate collection. However, the mPCR was able to type 100% of the tested isolates compared to IHA, which identified only 83% of the original collection and 77% of the additional collection. Therefore, it is perhaps more appropriate to consider that the *in silico* serovar is the new gold standard, in which case we estimate that the IHA serotyping method is only 72% accurate based on the concordance between the two methods and the total number of isolates tested. This takes into account the NT isolates and those with results that differ between the two methods. It is of course possible that IHA truly reflects the effective serovar of the bacterium when it is being tested in the laboratory, in that capsule gene expression might be off under these conditions, but we contend that the mPCR is more useful in these circumstances if the serotyping is being performed to obtain maximum information about the isolate and to help define disease potential.

Surveillance of this bacterium is focused on the isolates that are responsible for clinical disease cases, and only a single purified colony isolated from a case is usually serotyped, due to the expense of the current IHA test. This means that potential multiple infections (4, 29, 49) are not routinely monitored in pig herds (whether they are disease-associated or carriage isolates); therefore, the real prevalence of serovars may differ from that reported in the literature. In contrast, this mPCR can use a loopful of bacteria, whether from passaging a colony or using multiple colonies grown from a clinical sample that might contain colonies of different serovars; thus, we might be able to detect multiple serovars of *H. parasuis* at once from a single clinical sample. This mPCR would also allow the testing of multiple purified single colonies of *H. parasuis* that may be grown from a single clinical sample. These surveillance strategies would give more comprehensive figures for carriage rates and coinfection rates for the different serovars within individuals or at the herd level. The availability of molecular methods for detecting this fastidious organism in postmortem tissues would allow for more widespread application of the test and might enable a more accurate understanding of the true contribution of different serovars to clinical disease. For example, serovar-based differences in ease of culture and isolation may exist that can be overcome by new molecular detection methods. In the future, it may be also possible to detect *H. parasuis* in more accessible sample sites, such as the nasal cavity or oral fluid. This would pave the way for prospective sampling of herds. The removal of or reduction in cross-reactions will also make a clearer picture for the decision to vaccinate, particularly between serovars 4 and 7, which is a common cross-reaction in the United Kingdom. Rapid serovar identification will enable earlier introduction of prophylactic vaccination. However, the presence of multiple isolates of *H. parasuis* in the same animal (4, 29, 49) may mean that care needs to be taken when interpreting the results. There have been reports that some serovars are more virulent than others, for example, serovars 5 and 13 (1, 23, 50), and mPCR results will help determine whether or not multiple virulent serovars or a mixture of virulent and avirulent serovars is present in a sample.

In summary, we have developed a molecular serotyping mPCR that can differentiate 14 of the 15 serovars of *H. parasuis*. A total of 234 *H. parasuis* isolates from two isolate collections were tested using this new assay, and 100% of the isolates were serotypeable using the mPCR. There were no ambiguous cross-reactions between different serovars of *H. parasuis*, nor were there any cross-reactions with any other commensal or pathogenic bacteria tested to date. Of the isolates tested by mPCR, 12% had results that differed from the IHA serotyping assays (NT isolates excluded), and much of this variance is explained by previously discussed difficulties with the IHA method. Therefore, this molecular serotyping assay is a significant improvement on the current methods, reducing nontypeability, ambiguity, and cost of testing. The mPCR method described is fast, simple, and transferable to a molecular diagnostic laboratory with basic equipment, and it can be performed on crude gDNA derived directly from bacterial colonies.

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