Title: Development of a multiplex PCR for rapid molecular serotyping of *Haemophilus parasuis*

Kate J. Howell[1#] (kjh52@cam.ac.uk), Sarah E. Peters[1] (sep34@cam.ac.uk), Jinhong Wang[1] (jw401@cam.ac.uk), Juan Hernandez-Garcia[1] (jh937@cam.ac.uk), Lucy A. Weinert[1] (lucy.weinert@gmail.com), Shi-Lu Luan (shilu.luan@gmail.com), Roy R. Chaudhuri[2] (r.chaudhuri@sheffield.ac.uk), Øystein Angen[3] (Oystein.Angen@vetinst.no), Virginia Aragon[4] (Virginia.Aragon@cresa.uab.cat), Susanna M. Williamson[5] (Susanna.Williamson@apha.gsi.gov.uk), Julian Parkhill[6] (parkhill@sanger.ac.uk), Paul R. Langford[7] (p.langford@imperial.ac.uk), Andrew N. Rycroft[8] (ARycroft@rvc.ac.uk), Brendan W. Wren[9] (brendan.wren@lshtm.ac.uk), Duncan J. Maskell[1*] (djm47@cam.ac.uk) and Alexander W. Tucker[1*] (awt1000@cam.ac.uk) on behalf of the BRADP1T Consortium

[1]Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK.

[2] Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, UK.

[3]Norwegian Veterinary Institute, N-0106 Oslo, Norway

[4]Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193, Bellaterra, and Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain

[5]Animal and Plant Health Agency (APHA), Rougham Hill, Bury St Edmunds, Suffolk IP33 2RX

[6]The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK

[7]Section of Paediatrics, Department of Medicine, Imperial College London, St. Mary's Campus, London, W2 1PG, UK

[8]The Royal Veterinary College, Hawkshead Campus, Hatfield, Hertfordshire, AL9 7TA, UK

[9]Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street,

London, WC1E 7HT, UK

#Corresponding author

* These authors made an equal contribution to the manuscript.

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Abstract

Haemophilus parasuis causes Glässer's disease and pneumonia in pigs. Indirect haemagglutination (IHA) is used to serotype this bacterium, distinguishing fifteen serovars with some non-typeable isolates. The capsule loci of the fifteen reference strains were annotated and significant genetic variation was identified between serovars, with the exception of serovars 5 and 12. A capsule locus and *in silico* serovar was identified for all but two non-typeable isolates in our collection of over 200 isolates. Here we describe the development of a multiplex PCR, based on variation within the capsule loci of the fifteen serovars of *H. parasuis,* for rapid molecular serotyping. The 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 $4.29x10^5$ 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 *in silico* results. In addition, the two *in silico* non-typeable isolates were typeable using the mPCR. A further 84 isolates were analysed by mPCR and compared to IHA serotyping results with 90% concordance (excluding those non-typeable by IHA). The mPCR was faster, more sensitive, and more specific than IHA enabling the differentiation of fourteen of the fifteen serovars of *H. parasuis*.

Introduction

Haemophilus parasuis is a Gram-negative bacterium commonly found in the upper respiratory tract of the pig and, in 1910, it was identified 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 and septicaemia, as well as pneumonia (1–5). Based on statistics from the USA, *H. parasuis* is the leading cause of mortality (alongside the PRRS virus) in nursery herds, and is the third most important bacterial pathogen affecting finisher herds (6). *H. parasuis* also contributes to multi-factorial porcine respiratory disease complex, the leading cause of mortality in grower-finisher pigs in the USA (7). Diagnostic submissions to veterinary investigation centres of the Animal and Plant Health Agency (APHA) in 2013-14 recorded the highest annual rate 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 post-weaning and is associated with the loss of maternally-derived antibodies and the endemic presence of the bacterium in herds (1, 5).

The treatment and prevention of Glässer's disease is implemented via strategic delivery in feed or water of penicillin-based antimicrobials. On-going treatment may be administered to successive batches of susceptible pigs for several months after an outbreak to ensure the herd's full recovery (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 into and out of the herd is currently the best method of prevention, as it reduces the risk of introducing new strains (5, 14, 15).

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 to 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 sub-typing 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 fifteen serovars, with a considerable number of non-typeable (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 are contributing 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 whereas if samples are taken from the upper respiratory tract or the lung there is a higher chance of the isolate being a co-infection rather than the isolate responsible for the disease. This might well introduce sampling bias and the relative proportions of different serovars amongst 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 fifteen serovars of *H. parasuis* in 1992 using the gel immune-diffusion assay (GID) (23), which has since been superseded by an indirect haemagglutination assay (IHA) (30–32); this has increased the proportion of typeable strains from 60% to 80%. An isolate may be reported as non-typeable 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” reaction is chosen as the main serotyping result, but this can be dependent on a 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 accuracy of the serotyping of *H. parasuis* would aid the understanding of the epidemiology of this pathogen and allow optimization of vaccination strategies for 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 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 as 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 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. Analysis of the first complete *H. parasuis* genome sequence (strain SH0165) identified a 14kb polysaccharide biosynthesis region that was proposed to encode O-antigen, with twelve 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 that 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 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 non-typeable (NT) by IHA contained a capsule locus that matched one of the fifteen 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 they require further investigation.

Here we describe the design of a molecular serotyping PCR, based on variation within the capsule loci, capable of discriminating between fourteen of the fifteen 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 APHA from farms in England and Wales between 1993 and 2011, isolates from Denmark, Spain and Australia, as well as the fifteen serotyping reference strains. This collection included disease- and non-disease-associated isolates, all of which were genome 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 could be predicted *in silico* based on the capsule genes (44). A subset of 150 isolates from of this original collection was used for the validation of the mPCR, 117 of which had been serotyped by IHA and all of which a serovar had been predicted by *in silico* analysis. This subset included isolates representing all fifteen serovars and included those previously serotyped including those with cross-reactions (n=22), all non-typeable isolates (n=19) and a selection of isolates that have not been serotyped (n=33).

An additional 84 disease-associated isolates of *H. parasuis* were collected by the APHA during 2013 and 2014; we have called this the additional isolate collection. Sixty-six of these were serotyped by Innovative Veterinary Diagnostics (IVD), Germany, using IHA and were of a variety of serovars (serovar 1, 2, 4, 5, 6, 9, 13, 14, 15) as well as non-typeable isolates (n=15). Nine of the isolates had cross-reactions reported in their serotyping results. The remaining eighteen 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 it enabled 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 were also genome sequenced (European Nucleotide Archive: ERS132116, ERS132148, ERS132149, ERS132152-ERS132156, ERS132158-ERS132160, ERS132162-ERS132165, ERS132169, ERS132170). These genomes were evaluated using BLASTn against the primers designed for

155 *H. parasuis*, as they are from species that are most likely to cross-react. These isolates together with further
156 field isolates of *Actinobacillus pleuropneumoniae* (n=3), *Bordetella bronchiseptica* (n=1) and *Streptococcus*
157 *suis* (n=3), all common bacteria in the upper respiratory tracts of pigs, were used in this study as part of a
158 negative control panel for the mPCR.

159 *Species-specific marker design*

160 A Perl script was used to produce a draft core genome of *H. parasuis* using the genome sequences of the
161 original collection (n=212) (44). The protein-coding sequences from the published complete genome of *H.*
162 *parasuis* SH0165 (45) were compared using tBLASTn to the *H. parasuis* genomes. If a coding sequence had
163 80% identity over 80% of the length of the gene to a contig in all of the *H. parasuis* genomes, then the gene
164 was considered to be part of the "core" genome. This list represents the most conserved genes in the *H.*
165 *parasuis* genome. The SH0165 Fasta sequence for each gene from the core genome was compared to the
166 non-redundant (nr) NCBI database using BLASTn to identify genes with matches only to *H. parasuis*. These
167 core genes were also compared by BLASTn with the genome sequences of the other *Pasteurellaceae*
168 isolates. Genes with matches in *H. parasuis* only were taken forward as potential species-specific markers.
169 The alignments of the genes were assessed using alistat (46) to study the average alignment identity and
170 minimum alignment identity between two sequences in the alignment. Genes with greater than 95% average
171 alignment identity were chosen to create a shortlist of potential species-specific markers with a variety of
172 amplicon sizes.

173 *Serotyping mPCR Design*

174 The capsule loci of the fifteen serovar reference strains were previously sequenced and annotated (43). The
175 majority of isolates of the same serovar shared high levels of identity in their capsule loci, as expected (>95%
176 for the majority of serovars) (44). Where loci did not match within the same serovar, the majority of the
177 capsule loci matched to a recorded cross-reaction (44). Twenty-two isolates in the original isolate collection
178 had recorded cross-reactions, and four of these isolates matching to the minor reaction rather than the
179 dominant serovar in the cross-reaction results. A further ten isolates in the original isolate collection had
180 different results to the IHA serotyping result. The agreement between the IHA serotyping and the capsule
181 loci was tested using an un-weighted 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) and a shortlist of genes found in only one or two of the fourteen known capsule loci was made, from which to design primers for distinguishing the serotypes. The original capsule variation diagram from Howell et al. 2013 has been adapted to show the gene differences and target genes for the multiplex design (Figure S1).

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 40-60% 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 non-specific 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 re-designed 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 shortlist. A pair of primers was chosen for each gene, which would give approximately 20-50 bp separation between all amplicons when combined into an mPCR. Primers were obtained in dehydrated, de-salted form from Sigma-Aldrich (Haverhill, Cambridge). The final target genes and primers for the mPCR are shown in Table 1.

Primer optimisation

All primers were initially tested using gradient PCRs using One*Taq*® Quick-Load® 2X master mix with standard buffer (New England BioLabs) following the product specifications and protocols. Amplification of the targets was initiated at 94ºC for 30 seconds, followed by 30 cycles of 3-step cycling comprising denaturation at 94ºC for 30 seconds, annealing at a temperature range for the gradient PCR between 52-64ºC

for 30 seconds, 68ºC for 60 seconds for extension and a final extension of 68ºC for 5 minutes. Each PCR reaction contained 12.5µl One*Taq*® Quick-Load® 2X master mix, 0.25µl of each primer (at 20µM each), 2µl of gDNA for each isolate (at >10ng/µl) and 10µl UltraPure H_2O (Life Technologies) to a final volume of 25µl. 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. UltraPure H_2O was used as a negative control for all PCRs. Gel electrophoresis was performed in 2.0% agarose in 1x TBE with 5% Sybrsafe dye (Invitrogen), at 120V for 50 minutes using the Quick-Load® 100 bp DNA Ladder (New England BioLabs) as the molecular size standard. All results were analysed using a GelDoc^{TM} XR Imager (Bio-Rad). Each pair of primers was then tested on the panel of reference strains for the fifteen serovars (Table S1) using 25µl PCR reactions and the PCR protocol above with the consensus annealing temperature of 64ºC from the individual gradient PCRs. PCR purification was performed using a 50µl PCR reaction and the QIAquick PCR Purification Kit (Qiagen) as per the manufacturer's instructions. PCR products were sequenced using the Source Biosciences Sanger sequencing service. 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 50µM primer mix using 1xTE buffer. DMSO (Sigma-Aldrich) was added to the PCR reactions at 1% of the total reaction volume and the annealing temperature was reduced to 58ºC to improve the production of bands of equal intensity. The primer mixes were also optimised aiming for equal intensity for each amplicon, with a ratio of 1:0.25 serovar-specific primers to species-specific primers. The final PCR reaction mix included 12.5µl One*Taq*® Quick-Load® 2x master mix, 3µl of the primer mix, 2µl of gDNA for each isolate (at >10ng/µl) and 7.25µl UltraPure H_2O to a final volume of 25µl. Gel electrophoresis for the mPCR was extended to 90 minutes for better separation of the amplicons.

The mPCR was tested on 234 isolates (a subset of 150 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

235 results were compared to the *in silico* serovar predictions. For the additional isolate collection it was only
236 possible to compare the mPCR result to the IHA serotyping result if known.

237 Genomic DNA extraction requires pure culture, can be time consuming, and adds additional cost to the
238 diagnosis procedure and so colony PCR methods can be more convenient for diagnostic laboratories.
239 Therefore, for comparison, 20 isolates from the additional isolate collection were also tested using a colony
240 PCR method. A loopful of bacteria from a passaged plate of pure culture was resuspended in 50µl of
241 UltraPure H_2O, which was heated to 100ºC for 30 minutes, and centrifuged at 4,000 g for 1 minute before
242 the supernatant was used in the mPCR reaction. The same volume of supernatant was used in the mPCR
243 reaction as the volume that was used for pure genomic DNA. The results of mPCR were compared to those
244 of the IHA serotyping method (where available).

245 *Limit of detection of the mPCR*

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

251

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 had previously been determined as NT using the IHA method (44). Cohen's Kappa (47) was used to test agreement between the IHA and *in silico* analyses (for isolates where 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 (2,4) 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(2) identified as a serovar 4, three serovar 7 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 serotype by *in silico* analysis the serovar prevalence of the 117 isolates which had been serotyped by IHA in comparison with the *in silico* prediction of serotype of those isolates in the original collection was reassessed (Figure 1, (44)). From the original IHA serotyping results, serovars 5, NT, 4, 7 and 13 were the most prevalent serovars, in order of frequency. In comparison, the *in silico* results of all isolates showed unequivocally that serovar 4 was the most prevalent, followed by 5, 13 and 7 and none of the isolates was NT by *in silico* analysis, in comparison to 19 out of 117 by IHA serotyping.

The large amount of genetic variation between the capsule loci of the fifteen 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 less than 51% identity at the nucleotide level. The target genes amongst the variable region can be seen in Figure S1. 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 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 artefact 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, where 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 primers for serovar 2 were designed against a divergent *wzx* gene and the *amtA* gene was used to identify serovar 11 (Figure 2). By testing the serovar-specific primer pairs individually it was shown that each pair gave an amplicon of the expected size, each of which was only produced by the expected serovar (Figure 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 (sp-sp) marker, with an amplicon size of 275 bp, is shown in Figure 2. This sp-sp marker (HPS_219690793 - unknown function) was chosen from a shortlist of highly conserved genes from the core genome, as it fitted best with the serovar-specific amplicon sizes.

During the optimisation 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 specificity of the serotyping mPCR primers was 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* (Figure 3). Using DNA isolated from six reference strains of *H. parasuis* the average minimum concentration of DNA detectable 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 (Figure 4).

Validation of the serotyping multiplex

The molecular serotyping assay was validated using 150 isolates, covering all fifteen serovars, including 117 isolates that had been previously serotyped by IHA, including 19 isolates that were deemed non-typeable. 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 so was 100% accurate. A serovar could be 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 methods. 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 2013-2014, is shown in Table 3. Of the 66 isolates previously tested by IHA, in only 51 could a serotype be determined and 15 were classed as NT all of which could be 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 UK in the period 2013 - 2014, with serovar 5 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 but 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 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 their capsule structures or the composition of the antigens used in the IHA serotyping assay more closely. This might point, for example, to a difference in gene expression, rather than the presence or absence of an allele, as the determinant of the difference between 5 and 12 picked up by typing anti-sera. In the UK the Porcilis Glässer vaccine cross-protects (18) between serovar 5 and 12 and so no immediate negative consequences can be seen from the grouping of serovar 5 and 12 in this mPCR assay.

Conventionally, IHA serotyping would be considered the gold standard with which to compare our mPCR results. However, IHA serotyping has several well-known drawbacks (non-typeable isolates, cross-reactions, difficulties in producing anti-sera) 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 non-typeable isolates tested with the mPCR were assigned to a capsule type, with only 12% of isolates assigned to a different serovar than predicted by IHA excluding NT isolates. The majority of 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 latter as the "gold standard", then 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 isolates tested compared to IHA identifying 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 is truly reflecting 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 to 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 disease-associated or carriage); 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 of a colony or using multiple colonies grown from a clinical sample that could contain colonies of different serovars, and 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 co-infection rates for the different serovars within individuals or at the herd level. The availability of molecular methods for detection of this fastidious organism in post-mortem tissues would allow for more widespread application of the test, and might enable a more accurate understanding of the true contribution of different serotypes to clinical disease. For example, serotype-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 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 UK. 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 to 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 fourteen of the fifteen serovars of *H. parasuis.* A total of 234 *H. parasuis* isolates from two isolate collections were tested using

this new assay and 100% of 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 non-typeability, ambiguity and cost of testing. The mPCR method described is fast, simple and transferable to a molecular diagnostic laboratory with basic equipment and can be performed on crude gDNA derived directly from bacterial colonies.

Competing Interests:

The authors declare that they have no competing interests.

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List of consortium members: **University of Cambridge:** Duncan J. Maskell**,** Alexander W. (Dan) Tucker, Sarah E. Peters**,** Lucy A.Weinert**,** Jinhong (Tracy) Wang**,** Shi-Lu Luan**,** [§]Roy R. Chaudhuri. [§]Present address: Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, UK.**Royal Veterinary College:** Andrew N. Rycroft, Gareth A. Maglennon, Dominic Matthews. **Imperial College London:** Paul R. Langford**,** Janine T. Bossé**,** Yanwen Li. **London School of Hygiene and Tropical Medicine:** Brendan W. Wren, Jon Cuccui, Vanessa S. Terra.

Figure Legends:

Figure 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). Agreement between the IHA and in silico serovar prediction was tested using unweighted Cohen's kappa test, with p-value 0.0000128, excluding the NT isolates.

Figure 2: Band patterns for the molecular serotyping PCR for all 15 serovars of H. parasuis. M – Quick-Load 100 bp DNA Ladder (NEB), and S1-S15 represent the fifteen serovars of H. parasuis. Sp-sp denotes the species-specific marker.

Figure 3: Negative control panel showing specificity of the primer sets across a range commensal and pathogenic bacteria found in the pig respiratory tract (Haemophilus parasuis positive controls serovar 2 and serovar 5, Actinobacillus minor, A. porcinus, A. indolicus, Streptococcus suis, A. pleuropneumoniae, Bordetella bronchiseptica). M - Quick-load 100 bp marker (NEB) and H_2O as the negative control.

Figure 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 genome/µl is used. M - Quick-load 100 bp marker (NEB) and H_2O as the negative control.

Tables

Table 1: Serotyping multiplex primers and estimated product sizes

Gene	***Forward Primer 5' to 3'***	***Reverse Primer 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_2196907 93	ACAACCTGCAAGTACTTATCGGGAT	TAGCCTCCTGTCTGATATTCCCACG	All	275

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=78). All non-typeable (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.

Serovar by mPCR	**Serovar by IHA**																
	1	2	3	4	5 or 12	6	7	8	9	10	11	13	14	15	NT	Unknown	Total
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

Table 3: Summary of the mPCR serotyping results of the additional isolate collection of UK isolates from 2013 – 2014 (n=84). Cross-reactions in the IHA result were ignored for this comparison. All non-typeable (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.

Serovar	**Serovar by IHA**																
by mPCR	1	2	3	4	5 or 12	6	7	8	9	10	11	13	14	15	NT	Unknown	Total
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

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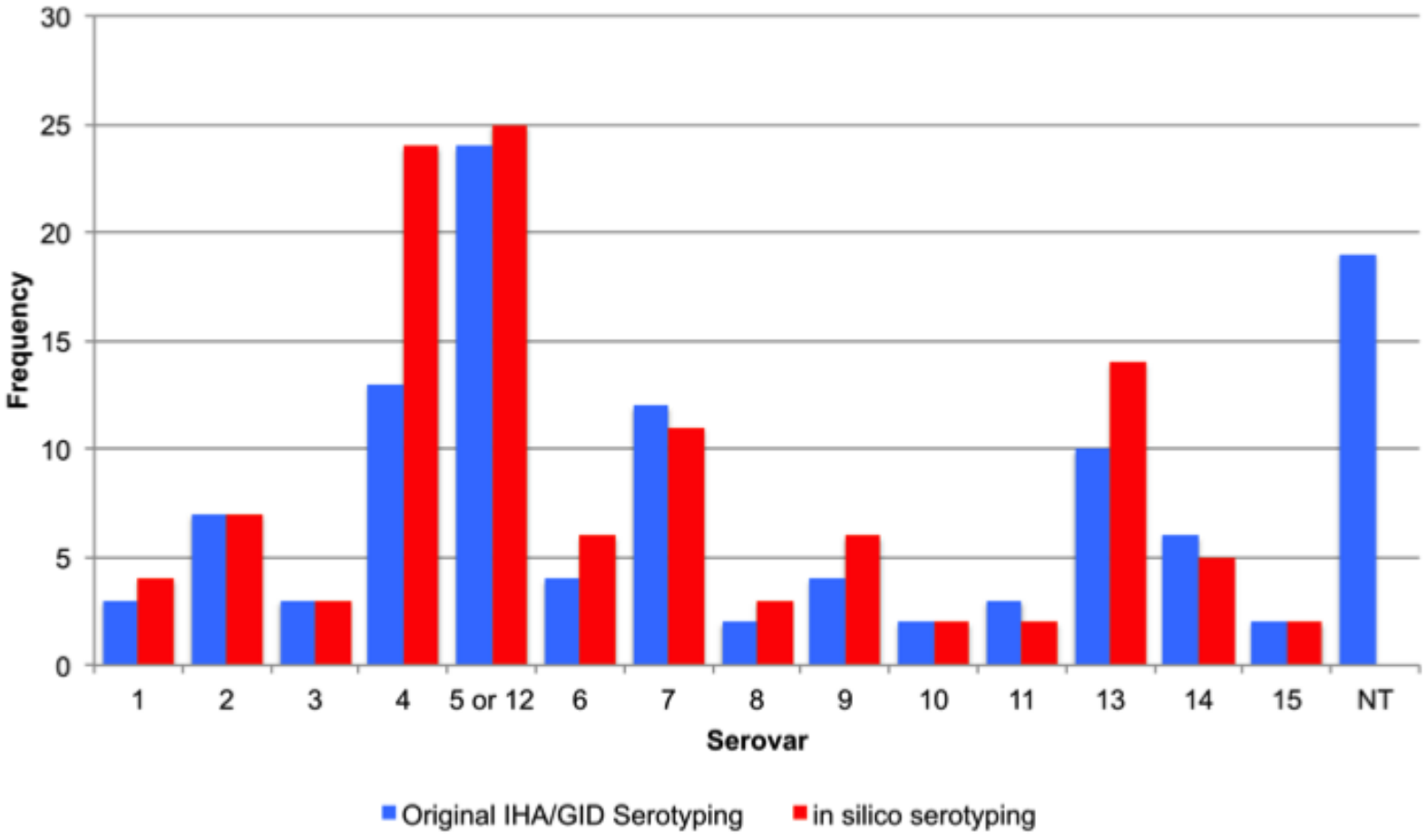
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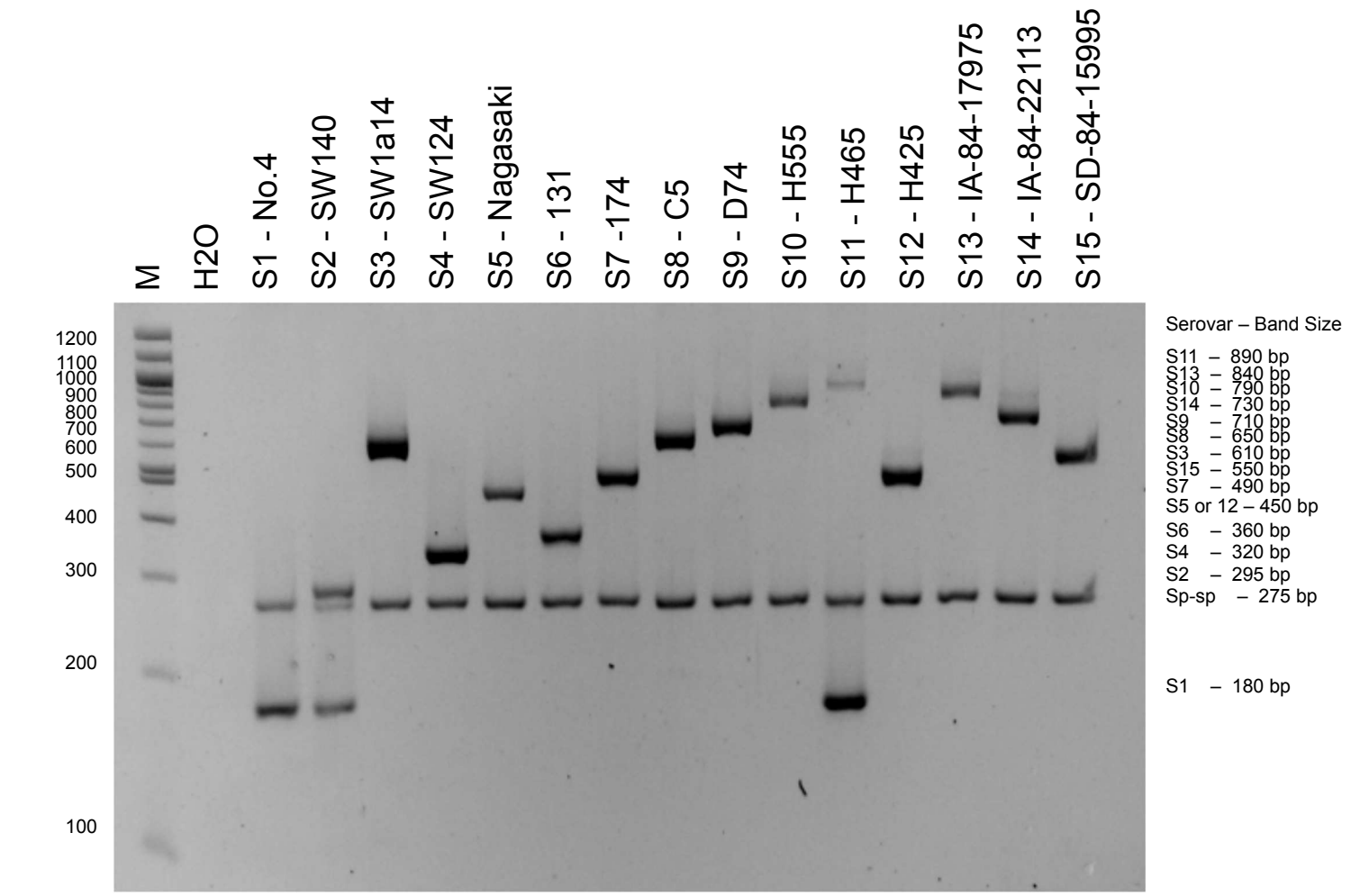
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S9 - D74
S10 - H555
S11 - H465
S12 - H425
S13 - IA-84-17975
S14 - IA-84-22113
S15 - SD-84-15995
1200
1100
1000
900
800
700
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500
400
300
200
100
Serovar – Band Size
S11 – 890 bp
S13 – 840 bp
S10 – 790 bp
S14 – 730 bp
S9 – 710 bp
S8 – 650 bp
S3 – 610 bp
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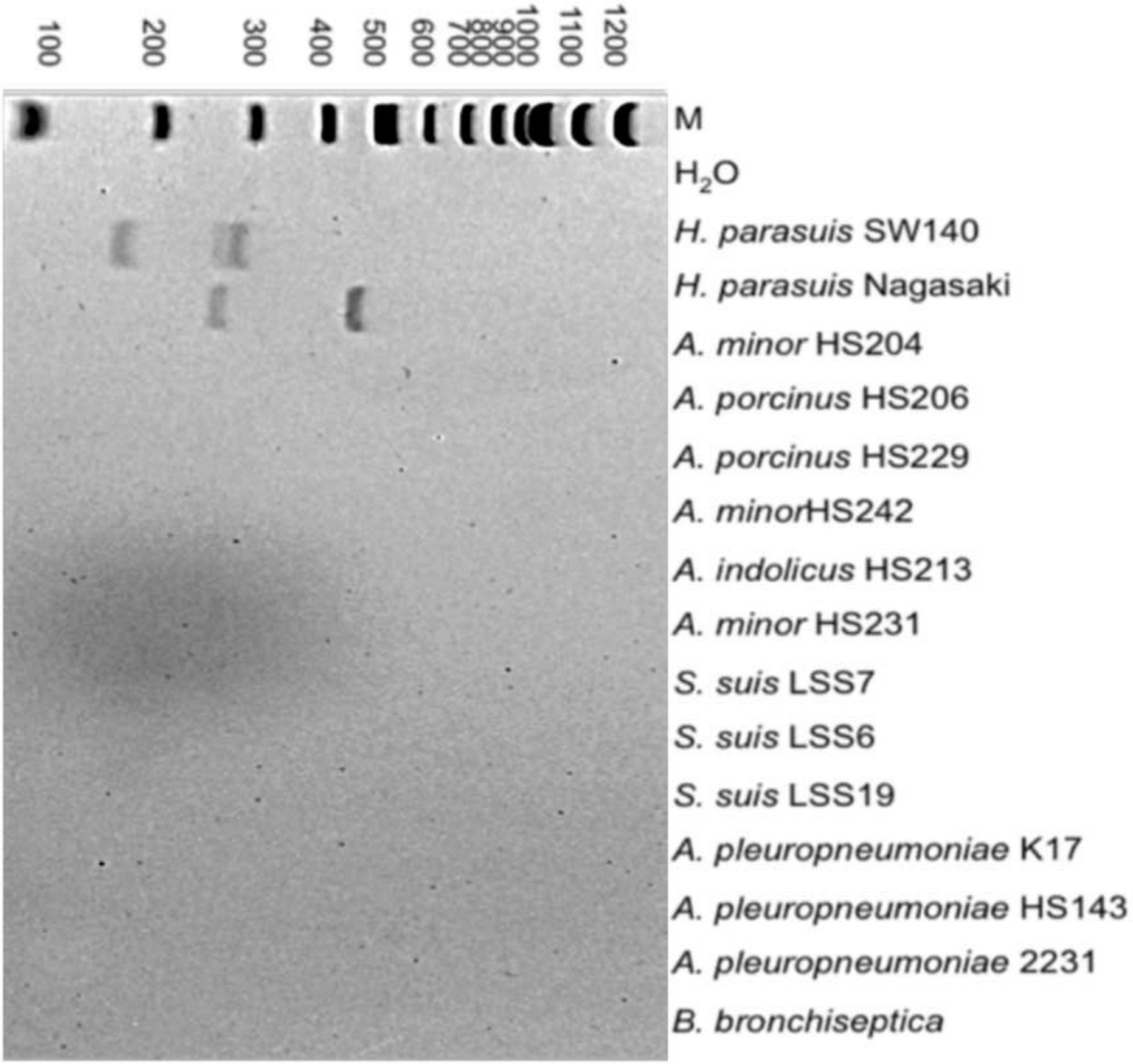
S1 – 180 bp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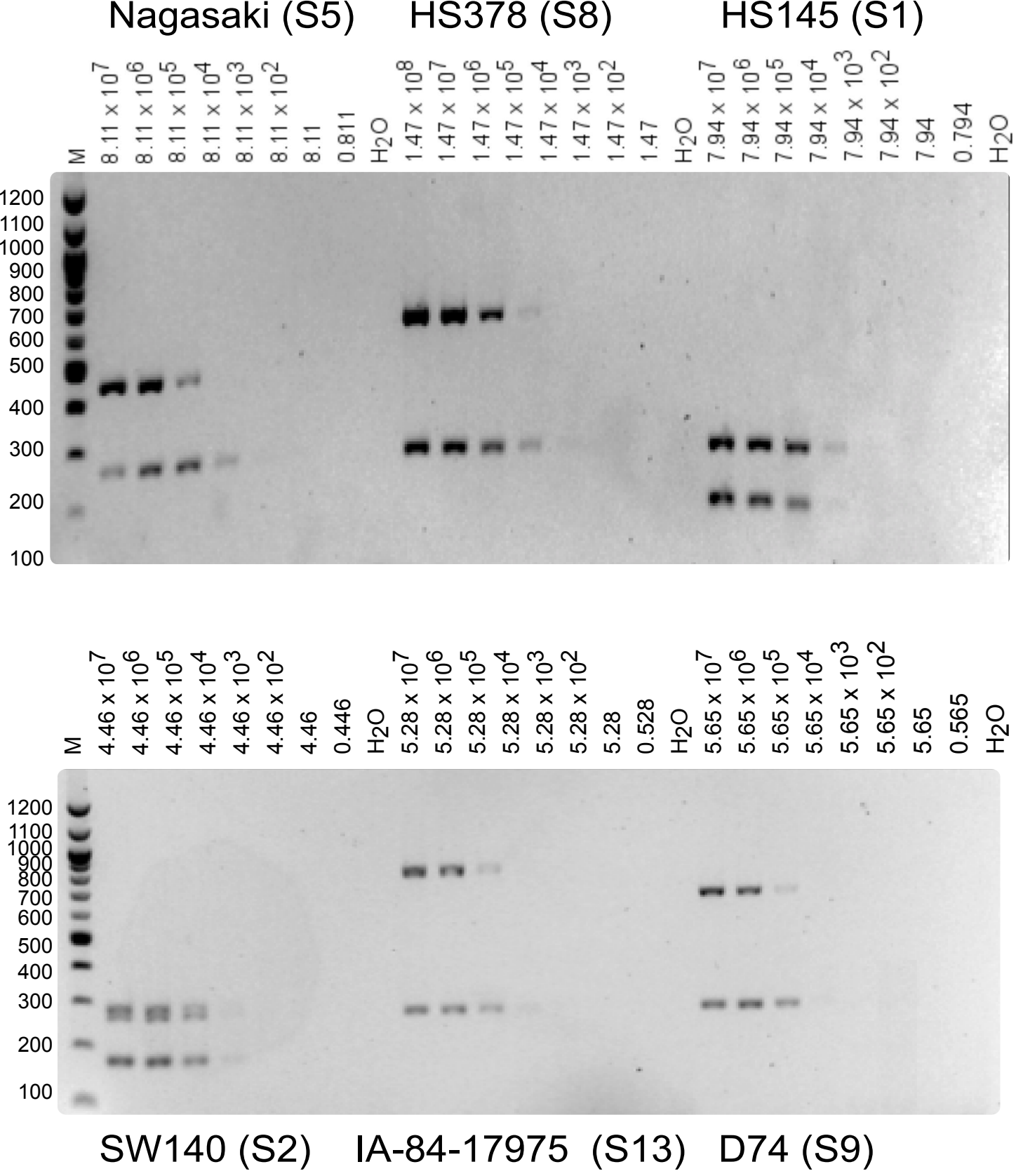
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1200
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100
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H2O
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5.65 x 10^6
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5.65
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H2O
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