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Multilocus Sequence Typing of the Porcine and Human Gastric Pathogen *Helicobacter suis*

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Helicobacter suis **is a Gram-negative bacterium colonizing the majority of pigs, in which it causes gastritis and decreased daily weight gain. *H. suis* is also the most prevalent gastric non-*Helicobacter pylori Helicobacter* species in humans, capable of causing gastric disorders. To gain insight into the genetic diversity of porcine and human *H. suis* strains, a multilocus sequence typing (MLST) method was developed. In a preliminary study, 7 housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC*) of 10 *H. suis* isolates cultured *in vitro* were investigated as MLST candidates. All genes, except the *ureI* gene, which was replaced by part of the *ureAB* gene cluster of *H. suis*, displayed several variable nucleotide sites. Subsequently, internal gene fragments, ranging from 379 to 732 bp and comprising several variable nucleotide sites, were selected. For validation of the developed MLST technique, gastric tissue from 17 *H. suis*-positive pigs from 4 different herds and from 1 *H. suis*-infected human patient was used for direct, culture-independent strain typing of *H. suis*. In addition to the 10 unique sequence types (STs) among the 10 isolates grown *in vitro*, 15 additional STs could be assigned. Individual animals were colonized by only 1 *H. suis* strain, whereas multiple *H. suis* strains were present in all herds tested, revealing that *H. suis* is a genetically diverse bacterial species. The human *H. suis* strain showed a very close relationship to porcine strains. In conclusion, the developed MLST scheme may prove useful for direct, culture-independent typing of porcine and human *H. suis* strains.**

Helicobacter suis is a Gram-negative, motile, tightly coiled, spiral-shaped, and microaerophilic bacterium that colonizes the gastric mucosa of the majority of pigs worldwide (1–3). Its reported prevalence depends on the study. Mostly, however, this bacterium is detected in more than 60% of pigs at slaughter age (1–4). *H. suis* infection in pigs is associated with chronic gastritis and decreased daily weight gain (5). In addition, associations have been made between a natural *H. suis* infection and the presence of ulcers in the pars oesophagea of the stomach (6–8). *H. suis* is also of zoonotic importance, as it is the most prevalent gastric non-*Helicobacter pylori Helicobacter* (NHPH) species in humans (4, 9). Pigs are considered to be an important source of infection for humans. Besides direct contact with animals, the consumption of raw or undercooked pig meat may also be a source of human infection (10, 11).

The first isolation of *H. suis* was described only in 2008 (12). In subsequent years, we gathered a total of 10 isolates, cultured *in vitro*, of this extremely fastidious microorganism. All isolates originate from pig stomachs, and until now, no isolates have been obtained from infected humans. To our knowledge, the above-mentioned *in vitro* isolates are the only ones available worldwide, and still now, *H. suis* isolation remains difficult and time-consuming. Often, mucus scrapings from half of a porcine stomach are needed to successfully isolate a new *H. suis* strain.

In order to gain insight into the strain diversity of both human and porcine *H. suis* strains, several typing methods can be used. However, given the extremely fastidious nature of this microorganism, a culture-independent method should be used, allowing typing of *H. suis* directly in stomach samples. Multilocus sequence typing (MLST), introduced in 1998, has been widely used in molecular epidemiology and population biology of bacterial species (13–16) and has proven its usefulness for typing strains of other *Helicobacter* species (17–19). In addition, this technique uses the unambiguousness and portability of nucleotide sequence data, which allows results from different laboratories to be compared without exchanging strains (20–22). Although it also has some drawbacks, including a relatively high cost (21), MLST is considered a gold standard for strain typing of bacterial species.

Our aim was thus to develop a robust *H. suis* MLST technique which can be applied to biological tissue without the need for cultivation.

MATERIALS AND METHODS

H. suis **isolates cultured *in vitro* and DNA extraction.** *H. suis* strains HS1 to HS10 were isolated from the gastric mucosa of sows as described previously (12). Bacteria were grown microaerobically (85% N_2, 10% CO_2, 5% O_2; 37°C; 48 to 96 h) on biphasic brucella (Becton Dickinson, Franklin Lakes, NJ) culture plates (with a pH adjusted to 5) supplemented with 20% fetal calf serum (HyClone, Logan, UT) and Vitox supplement (Oxoid), as described previously (23). Bacterial genomic DNA of all *H. suis* strains was extracted as described by Wilson (24).

Development of a *Helicobacter suis* multilocus sequence typing method. PCR assays were performed on a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). Different DNA polymerases (*Taq*, *Pwo*, and Accuzyme) were used in this study depending on the application. PCR products were first purified using a MSB Spin PCRapace kit (STRATEC Molecular GmbH, Berlin, Germany). Purified PCR products were sequenced using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA), and sequences were determined on an automatic DNA sequencer (ABI Prism 3100 genetic analyzer; Applied Biosystems). The electropherograms were exported and converted to Kodon software (Applied Maths, Sint-Martens-Latem, Belgium).

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TABLE 1 Oligonucleotide primers used for amplification and sequencing of complete coding sequences

Primer	Sequence (5′ to 3′)	Polarity	Amplicon size (bp)	Position (bp)	Annealing temp (°C)	Use of primer[a]
atpA JunF1	TTGTTAAAATTAAAAGCAGAAGAAATTAGCG	Forward	1,512	1–31	54	A, S
atpA JunR1	CTAGAGAGTTAGACTTAGTTTAAATTCTTCAATCGC	Reverse		1477–1512		A, S
ATPA-F	CCCCCCTATTCTCCCTTGCCC	Forward		1270–1290		S
ATPA-R	CAAGCCCCTATCGCCGGTATC	Reverse		169–189		S
AtpA_fw_in	TACCCGTAGGCGATGCGGTC	Forward		293–312		S
efp JunF1	ATGGCCATTGGAATGGGTGAAC	Forward	567	1–22	53	A, S
efp JunR1	TTAAACTTTGAGCTTTTCTAAATACTCC	Reverse		540–567		A, S
EFP-F	GCCTAATTTTAGAGGGGATAC	Forward		414–434		S
EFP-R bis	ATTAGGCTCTTCGCACTTATCC	Reverse		174–195		S
mutY JunF1	ATGTCCCAGCAAACCCAAATTGAGGTTG	Forward	873	1–28	58	A, S
mutY JunR1	CTACAAAATCCCCGTCTTTTCTAGTAATACC	Reverse		843–873		A, S
MUTY-F	AGTACCGCGTGCAAGTTTGGC	Forward		719–739		S
MUTY-R	CACGGGCATAATAACCTAGAC	Reverse		125–145		S
ppa JunF1	ATGAACATCTCAAAAATTCCAGTTAGTAACGC	Forward	528	1–32	56	A, S
ppa JunR1	TCAACCTTGGGCTTGTTGGTAATTTGCAAGTGC	Reverse		496–528		A, S
PPA-F2	CATTGTGCTACAAAAGATCAGAC	Forward		378–400		S
PPA-R2	TAGCTGGATATACCATAGATG	Reverse		134–154		S
trpC JunF1	ATGCATGATTTTTTAACAACCATGTTAGAA C	Forward	1,359	1–31	57	A, S
trpC JunR1	TCAATACTCCCGTAACATTTTTGCAATTTGTGC	Reverse		1327–1359		A, S
TRPC-F	GCTAGCTGGAGGGTTAAATGC	Forward		1209–1229		S
TRPC-R	GAGGGGGAGGCTTGTTTGCAC	Reverse		150–170		S
TRPC_rv_in	TTTAAGCGCAGTTGGCATGCA	Reverse		960–980		S
ureI JunF1	ATGCTAGGACTTGTGTTATTGTATGTTGCGATCG	Forward	588	1–34	55	A, S
ureI JunR1	TTACACCCAGTGTTCGATAAAGAGAAGCCAAGCAGG	Reverse		553–588		A, S
UREI-F	ACTGGTTGGATTGAGGAAGTGG	Forward		463–484		S
UREI-R	CAAACAATAGAGATCCCACCTAC	Reverse		106–128		S
yphC JunF1	ATGCTTAAAATTGCTATTTTGGGCAAGCC	Forward	1,317	1–29	57	A, S
yphC JunR1	TCATGTTTGCAAAGTTTCTTCTTTGCCTCTGGC	Reverse		1285–1317		A, S
YPHC-F2	TTGCTTTGGTTATGAATCGCCCTAAG	Forward		1175–1200		S
YPHC-R	AAAGCTCCACCCCATGGCCTGCTAG	Reverse		136–160		S

[a] A, primer used for amplification; S, primer used for sequencing.

Based on the whole genomic DNA sequence of *H. suis* strains 1 and 5 (25), seven housekeeping genes used for *H. pylori* multilocus sequence typing (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC*) were initially selected for the design of a multilocus sequence typing method for *Helicobacter suis*. The complete coding regions of these genes were amplified from 10 *H. suis* isolates cultured *in vitro* using *Pwo* polymerase with 3′-to-5′ proofreading exonuclease activity. Final reaction mixtures containing a total of 25 μl were prepared in 2 parts. Part 1 contained 6.25 μl deoxynucleotide triphosphates (dNTPs) (1 mM each), 0.3 μl of each primer at a concentration of 0.6 pmol/μl, 5.15 μl Aq HPLC, and 0.5 μl DNA sample. Part 2 included 0.25 μl *Pwo* DNA polymerase (5-U/μl stock solution), 2.5 μl 10× buffer containing $MgSO_4$, and 9.75 μl Aq HPLC. Briefly, following an initial 3-min denaturation at 94°C, amplification was done by 10 cycles of 94°C for 30 s, 53°C to 58°C for 30 s, and 72°C for 90 s, followed by 25 or 30 cycles of 94°C for 30 s, 53°C to 58°C for 30 s, and 72°C for 90 s, with an additional 5-s elongation time for each cycle. A final extension step was performed at 72°C for 10 min. Alternatively, amplification was done using Accuzyme DNA polymerase. Final reaction mixtures (10 μl) included 5 μl Accuzyme mix, 0.1 μl of each primer at a concentration of 0.5 pmol/μl, 3.8 μl Aq HPLC, and 1.0 μl DNA sample. Briefly, following an initial denaturation for 3 min at 94°C, amplification was done by 10 cycles of 94°C for 30 s, 53°C to 58°C for 30 s, and 72°C for 90 s, followed by 25 or 30 cycles of 94°C for 30 s, 53°C to 58°C for 30 s, and 72°C for 90 s, with an additional 5-s elongation time for each cycle. A final extension step was performed at 72°C for 20 min. Purification of PCR products and sequencing were performed as described above. Primers used for amplification and sequencing, as well as annealing temperatures, are shown in Table 1.

For all genes except the *ureI* gene, variable sites were detected. Based on the position of these sites, internal gene fragments (450 to 732 bp) were selected (Table 2). Amplification was done using *Pwo* or Accuzyme DNA polymerase with 3′-to-5′ proofreading activity. Primers used for amplification and sequencing of these variable regions are shown in Table 2. Briefly, following an initial denaturation for 3 min at 94°C, amplification was done by 10 cycles of 94°C for 30 s, 59°C to 69°C for 30 s, and 72°C for 90 s, followed by 25 or 30 cycles of 94°C for 30 s, 59°C to 69°C for 30 s, and 72°C for 90 s, with an additional 5-s elongation time for each cycle. A final extension step was performed at 72°C for 20 min. Purification of PCR products and sequencing were performed as described above. Based on the multiple alignment, using Kodon software, of the *ureAB* gene cluster of different *H. suis* strains (HS1, HS5, and NCBI accession numbers AF507995, AF508001, AF508008, AF508009, and AF508013), variable sites were detected in this gene cluster. Therefore, primers were designed for amplification and sequencing of the variable region of this gene cluster (Table 2).

Based on the nucleotide sequence of the internal gene fragments, allele numbers for each gene were assigned for isolates HS1 to HS10, cultured *in vitro*. The sequence type (ST) was defined by the combined allelic profile of the 7 alleles.

Validation of *Helicobacter suis* MLST. For validation of the developed MLST on tissue samples, gastric biopsy specimens were collected from slaughter pigs from 4 different herds. For each stomach, three pieces of tissue (1 cm^2; 1 from the corpus, 2 from the antrum) were collected using sterile biopsy punches. In addition, an antral gastric biopsy specimen from a pig veterinarian, suffering from gastric complaints and diagnosed with *H. suis* infection (M. Joosten, B. Flahou, T. Meyns, A. Smet, J. Arts, L. De Cooman, F. Pasmans, R. Ducatelle, and F. Haesebrouck, submitted for publication), was included as well. DNA was extracted using the Isolate genomic DNA minikit (Bioline, London, United Kingdom)

TABLE 2 Oligonucleotide primers used for *H. suis* MLST

Primer	Sequence (5′ to 3′)	Polarity	Amplicon size (bp)	Position (bp)	Annealing temp (°C)	Use of primer[a]
atpA-MLST-A	TTATGAGGTGGTTGAATTTGATACCGGC	Forward	790 (732)[b]	150–177	63	A, S
atpA-MLST-B	AGAGCCTGCCCCTTTCTTATCACTCATTT	Reverse		911–939		A, S
atpA-MLST-C	ATGATTGCATCAATGGCAACAGTGG	Reverse		530–554		S
efp-MLST-A	TACAAGGCGTTCCTTATCGCATTGT	Forward	470 (379)[b]	47–71	61	A, S
efp-MLST-B	CACCTCCCCCTCTAGCACATGG	Reverse		495–516		A, S
efp_mlstAquinto[c]	GGCCTTTGTACGGGCTAAA	Forward	379	105–123	58	A, S
efp_mlstBbis[c]	CACCACTGCCCCGGT	Reverse		469–483		A, S
mutY-MLST-A	CGCCCCTTTAGACCGGGTTTTACTT	Forward	650	90–114	61	A, S
mutY-MLST-B	GCCAAACTTGCACGCGGTACTTG	Reverse		717–739		A, S
mutY-MLST-C	TTAGGCAAAATGTGGGCGTGCTAGA	Forward		278–302		S
ppa-MLST-A	TGCCGTTATTGAAATCCCGTATGGA	Forward	480	45–69	60	A, S
ppa-MLST-B	CCTTGGGCTTGTTGGTAATTTGCAA	Reverse		500–524		A, S
trpC-MLST-A	TGTGGCCTTAAGCGGGTTAAAGATG	Forward	450	769–793	60	A, S
trpC-MLST-B	TCCAGCTAGCATAAAGCGATGGGAT	Reverse		1194–1218		A, S
ureAB_mlstA	GTGCGCTTTGAACCTGGCG	Forward	688 (676)[b]	523–541	69	A
ureB_mlstB	CCTGTTCCGCCTCCAAGCAT	Reverse		1191–1210		A, S
ureB_mlstA	ATGTATGGCCCCACTACAGGCG	Forward		759–780		S
yphC-MLST-A	GGATACAGGCGGGTTTGATGCAG	Forward	850 (717)[b]	162–184	59	A, S
yphC-MLST-B	TTTGATTTGGAGGATATGGCGCTTAGA	Reverse		985–1011		A, S
yphc-MLST-C	AAATGCCCTGATAGAGCAAGAACGC	Forward		579–603		S
yphC_mlstAtris[c]	AAAATCCCCCCACAAGATGAGGATAA	Forward	717	268–293	62	A, S
yphC_mlstBtris[c]	GATAGCACTTGTTGTAAGAAGCG	Reverse		962–984		A, S

[a] A, primer used for amplification; S, primer used for sequencing.
[b] The number in parentheses represents the actual length, after trimming, of nucleotide fragments used for multiple alignment, determination of ST, and generation of concatenated sequences.
[c] Primer pair used for amplification and sequencing of internal *efp* and *yphC* genes from the human *H. suis* strain described in this study.

according to the manufacturer's instructions. All DNA samples were screened for the presence of *H. suis* using a *Taq* polymerase-based species-specific PCR, as described by De Groote et al. (7). Three to six *H. suis*-positive samples/herd, as well as the human sample, were selected for multilocus sequence typing of colonizing *H. suis* strains, as described above.

Amplification of the internal gene fragments for the *efp* and *yphC* genes from the human *H. suis* strain, however, yielded no pure PCR products. Therefore, new primer pairs were designed, resulting in the amplification of a single PCR product of the expected size (Table 2). In order to enable comparison, internal *efp* and *yphC* gene fragments from *in vitro*-cultured *H. suis* strains and the porcine and human gastric tissue samples, obtained as described above, were all trimmed to this new size (379 bp and 717 bp for *efp* and *yphC*, respectively). Contig assembly of sequences obtained using different primers was done using Kodon software. The obtained sequences of internal gene fragments (harboring variable nucleotide sites) for individual genes were aligned using the CLUSTAL W program.

In order to assess the stability of allelic polymorphisms during *in vitro* culture of *H. suis*, strains HS1 and HS5 were cultured and passed onto new plates continuously for another 6 weeks. These long-term *in vitro*-cultured strains were designated HS1p17 and HS5p21, respectively. DNA was extracted and used for sequencing of the internal gene fragments as described above.

Based on the nucleotide sequence of the internal gene fragments, allele numbers for each gene were assigned for *H. suis* strains detected in porcine and human stomach tissues. The sequence type (ST) was defined by the combined allelic profile of the 7 alleles.

The relationship among the different *H. suis* strains was examined using the concatenated internal gene fragment sequences of the 7 MLST loci. A phylogenetic tree was constructed using the neighbor-joining method via the PHYLIP 3.69 package, using DNADIST for distance analysis (26).

RESULTS

Selection of genes for multilocus sequence typing. A total of 6,744 bp, representing the complete coding sequences of 7 housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC*), were amplified and sequenced for all *in vitro H. suis* isolates available (HS1 to HS10), revealing a unique concatenated sequence for all isolates. Fifty-six nucleotide sites (56/6,744; 0.83%) were found to be polymorphic. The *mutY* gene was shown to have the highest discriminatory power, with 32 variable nucleotide sites. The *ppa* gene showed 9 variable nucleotide sites, whereas the *yphC* gene revealed 5, the *atpA* gene revealed 4, and the *efp* and *trpC* genes revealed 3 variable nucleotide sites. Based on the complete *ureI* coding sequence (strains HS1 to HS10), this gene was shown to lack discriminatory power (0 variable nucleotide sites).

MLST analysis of *in vitro*-isolated *H. suis* strains and *H. suis* strains present in porcine and human gastric tissue. Based on the complete gene sequences, as described above, internal gene fragments/variability regions (379 to 732 bp) were selected for all 7 loci except the *ureI* gene. Instead, the latter was replaced by part of the *ureAB* gene cluster. For porcine *H. suis* strains HS1 to HS10, allele numbers and sequence types (STs) were assigned, as shown in Table 3. Determination of allele numbers for long-term *in vitro*-cultured HS1p17 and HS5p21 showed no differences for HS1p17 compared to its parental strain, HS1. For HS5p21, however, a new allele type was attributed for *mutY* and *trpC*, indicating that long-term *in vitro* culture, and possibly also *in vivo* colonization, may result in limited genetic diversification.

In addition, direct *H. suis* MLST strain typing was performed on gastric tissue of 1 *H. suis*-infected human and 17 *H. suis*-positive pigs at slaughter age from 4 different herds, as determined by

TABLE 3 Determination of allele numbers and sequence types for 10 *H. suis* strains isolated *in vitro* and *H. suis* bacteria present in 1 human and 17 porcine gastric tissue samples

Sample[a]	ST	Sample origin	Allele no.						
			atpA	*efp*	*mutY*	*ppa*	*trpC*	*ureAB*	*yphC*
HS1	1	*In vitro* culture	1	1	1	1	1	1	1
HS1p17	1	*In vitro* culture	1	1	1	1	1	1	1
HS2	2	*In vitro* culture	2	1	2	1	2	1	2
HS3	3	*In vitro* culture	1	2	2	1	2	1	2
HS4	4	*In vitro* culture	3	2	3	1	3	1	3
HS5	5	*In vitro* culture	4	2	4	1	1	1	1
HS5p21	6	*In vitro* culture	4	2	5	1	2	1	1
HS6	7	*In vitro* culture	1	3	6	1	1	1	3
HS7	8	*In vitro* culture	1	3	7	1	1	1	3
HS8	9	*In vitro* culture	1	4	8	1	1	1	1
HS9	10	*In vitro* culture	4	2	9	1	1	1	1
HS10	11	*In vitro* culture	1	4	3	2	1	1	3
A-P29	12	Herd A	4	2	9	1	2	1	1
A-P49	13	Herd A	1	4	10	1	1	1	3
A-P63	12	Herd A	4	2	9	1	2	1	1
B-V16	11	Herd B	1	4	3	2	1	1	3
B-V37	14	Herd B	1	2	3	1	1	2	4
B-V57	15	Herd B	1	2	3	1	1	1	1
B-V85	16	Herd B	1	2	3	1	1	2	3
C-P1A1	17	Herd C	1	2	11	1	1	1	1
C-P3A1	17	Herd C	1	2	11	1	1	1	1
C-P8A1	17	Herd C	1	2	11	1	1	1	1
C-P9A1	18	Herd C	1	2	12	1	1	1	1
D-P21A1	19	Herd D	1	2	13	1	2	1	5
D-P26A1	20	Herd D	1	2	14	1	1	1	2
D-P27A1	21	Herd D	1	4	15	1	1	1	1
D-P28A1	22	Herd D	1	3	16	1	2	1	2
D-P29A1	23	Herd D	1	2	16	1	2	1	2
D-P30A1	24	Herd D	1	2	14	1	1	1	6
HA	25	Human	1	2	17	1	1	1	1

[a] HS1 to HS10, *H. suis* isolates cultured *in vitro*; HS1p17 and HS5p21, long-term (6-week) *in vitro* cultures originating from HS1 and HS5, respectively; A-P29, for porcine gastric tissue samples, the number of the stomach (P29 to P63; V16 to V85; P1A1 to P30A1) is preceded by a letter designating the herd (A to D); HA, *H. suis* strain colonizing the antrum of a human patient suffering from reflux esophagitis and dyspepsia.

H. suis-specific PCR. Three stomachs from herd A were tested, 4 each from herds B and C were tested, and 6 stomachs from herd D were tested. For MLST analysis, selected variability regions were amplified and sequenced. Close examination of the electropherograms indicated that only 1 ST colonized each animal. For the human sample, as well as all 17 porcine gastric tissue samples, a total of 4,084 bp were aligned to the same gene regions of *H. suis* strains HS1 to HS10. In total, 48 nucleotide sites (48/4,084; 1.18%) were shown to be polymorphic. The *mutY* gene revealed a total of 27 variable nucleotide sites, whereas 7, 4, 3, 3, 2, and 2 variable nucleotide sites were detected in the *ppa* gene, *yphC* gene, *atpA* gene, *efp* gene, *trpC* gene, and *ureAB* gene, respectively. Twenty-five sequence types (STs) were assigned based on unique allele combinations (Table 3). In herd A, 2 out of 3 strains shared the same ST (ST11), while a third strain displayed an unrelated ST, belonging to another group (Fig. 1). In herd C, three out of four strains shared the same ST. For herds B and D, however, all strains showed a different ST. All data have been made available at http://pubmlst.org/hsuis/, developed and hosted at the University of Oxford (26a).

Relationships between the isolates. A neighbor-joining tree (unrooted) was constructed based on the concatenated sequences of all 7 MLST gene fragments (4,084 bp), indicating the genetic relationship between all strains tested (Fig. 1). In general, calculated bootstrap values were low (<70), resulting in the assignment of 4 groups, of which groups 1 and 2 were singletons. Although most strains detected in porcine gastric tissue belonged to the same group with low internal bootstrap values (group 4), strains belonging to a particular herd tended to cluster, indicating a lower genetic distance between strains present in the same herd. Figure 1 clearly shows that the human *H. suis* strain is closely related to porcine *H. suis* strains.

DISCUSSION

Up to now, very little is known about transmission of *H. suis* between pigs and from pigs to humans. It is thought that pigs are the most important source of infection for humans through direct contact with the animals or through the consumption of raw or undercooked pig meat contaminated with *H. suis* (10, 11). In addition, few data are available on strain-related differences in virulence or the immune response evoked in its animal or human host. So undoubtedly, there is a strong need for a robust *H. suis* strain typing method, which is essential to provide answers to some of the above-mentioned questions.

Baele et al. (12), describing the first successful *in vitro* isolation of *H. suis*, performed genomic fingerprinting of isolates HS1, HS2,

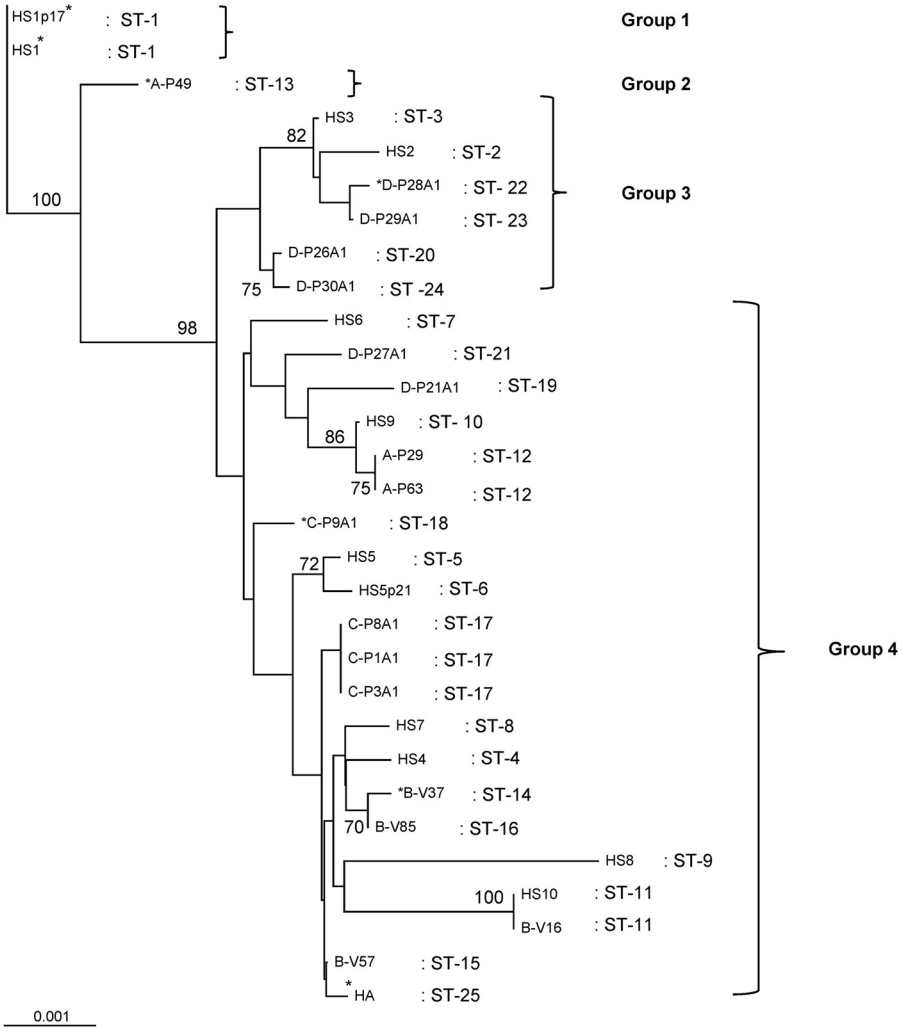

FIG 1 Neighbor-joining phylogenetic tree (unrooted), constructed using the concatenated sequences (4,084 nucleotides) of 7 *H. suis* gene fragments from 27 *H. suis* strains tested. Bootstrap values equal to or greater than 70 are presented at the nodes of the phylogenetic tree. The topology shows that all sequence types cluster into four groups. HS1* to HS10, *H. suis* isolates cultured *in vitro*; HS1p17* and HS5p21, long-term (6-week) *in vitro* cultures originating from HS1 and HS5, respectively; *A-P49, for gastric tissue samples, the number of the stomach (P29 to P63; V16 to V85; P1A1 to P30A1) is preceded by a letter designating the herd (A to D); *HA, *H. suis* strain colonizing the antrum of a human patient suffering from reflux esophagitis and dyspepsia.

and HS3 by amplified fragment length polymorphism (AFLP), revealing distinct AFLP patterns for these 3 isolates. Although this typing method has proven its value for strain typing of isolates cultured *in vitro* of several bacterial species (27, 28), this cannot be used for typing of *H. suis* strains in a complex matrix, such as biological tissue samples. Keeping in mind that *H. suis* isolation remains extremely difficult and time-consuming, we decided to develop an MLST technique which can be applied on samples without the need for *in vitro* cultivation. As a genotyping method and technique, MLST has often been used in strain typing studies and can be considered a gold standard (15, 18, 20–22).

For MLST analysis of strains of *H. pylori*, a closely related major human gastric pathogen, 7 housekeeping genes are used (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *yphC*, and *ureI*) (17, 19). The whole-genome sequences of *H. suis* strains 1 and 5 revealed the presence of a homologue for all of these genes (25). Therefore, these genes were also investigated as MLST candidates in our preliminary study. Each gene was successfully amplified and sequenced from all *H. suis* strains. The *mutY* gene was found to have, by far, the most discriminatory power, revealing 17 alleles for 30 samples tested. The remaining 6 genes revealed a significantly lower number of alleles than the *mutY* gene. The *ureI* gene was even found to have no variable nucleotide sites, and this locus was therefore replaced by part of the *ureAB* gene cluster in the final MLST scheme.

For *H. pylori*, all 7 loci used for multilocus sequence typing show a similar discriminatory ability, in contrast to what we found for *H. suis* (http://pubmlst.org/helicobacter/). Nevertheless, assessment of the allelic profiles for the 10 available *H. suis* isolates cultured *in vitro* enabled us to discriminate 10 distinct sequence types (STs), underlining the suitability of the proposed MLST scheme to identify different *H. suis* strains. In addition, this scheme was applied to gastric biopsy specimens from 1 human patient and 17 slaughter pigs originating from 4 different herds. We were able to distinguish 14 additional STs, and all tissue samples revealed the presence of only 1 ST, based on the analysis of electropherograms. Because isolation and purification of coloniz-

ing *H. suis* strains were not attempted on these samples, we cannot exclude the presence of additional sequence types in 1 individual animal at a lower colonization rate than that of the dominant strain. For *H. pylori*, it has been shown that mixed infections with more than 1 strain in single individuals are indeed common in certain geographic regions. These mixed infections are, in fact, important for genetic diversification, since *H. pylori* genomes seem more stable in the absence of a mixed infection in one individual (29). On the other hand, long-term *in vitro* culture of *H. suis* strain HS5 resulted in the appearance of new alleles for a number of genes, indicating that changes or mutations do occur during *in vitro* culture and most likely also during *in vivo* colonization, as has been described for *H. pylori* in the absence of mixed infection (30). Recombination events in the presence of other *H. suis* strains can be excluded, since *H. suis* strains HS1 and HS5 were purified before the onset of the present study from a single colony on 1% brucella agar plates after initial isolation from slaughterhouse pigs by biphasic culture methods (12).

Based on the neighbor-joining tree and calculated bootstraps of concatenated sequences from all 7 housekeeping genes, four groups were distinguished among the isolates tested in this study, of which 2 (groups 1 and 2) were singletons. Most *H. suis* strains belonged to a large group (group 4) with low internal bootstrap values, although strains belonging to a particular pig herd showed a tendency to cluster. For 2 out of 4 herds (B and D) tested in the present study, all samples revealed the presence of a different ST, indicating a substantial genetic heterogeneity among *H. suis* bacteria present at the herd level. The human *H. suis* strain clearly showed a close relationship to porcine *H. suis* strains, indicating that the patient, a pig veterinarian, most likely contracted the infection through its close contact with pigs (Joosten et al., submitted).

At this point, no conclusions can be drawn on possible correlations between STs and pathologies in pigs and humans. For the slaughterhouse samples, no detailed histopathology was performed. *H. suis* strains HS1 to HS9 have all been shown to generate an immune response and gastritis *in vivo* (4, 23, 31). Strain HS1 generates an immune response that is somewhat different from that of most other *in vitro*-cultured *H. suis* strains with regard to the expression of interleukin 4 (IL-4) and IL-6 (31), and interestingly, this strain constitutes a distinct singleton in the present study. Future studies should try to elucidate whether additional correlations can be made between the ST and the clinical significance of the strain.

In conclusion, we developed a multilocus sequence typing scheme and method for identification of *H. suis* strain differences, both in pure *in vitro* cultures and in biological samples. Most likely, this technique will further prove its usefulness, since until today, *H. suis* isolation and cultivation remain a challenge. In the future, this scheme can be used for strain typing, not only of porcine *H. suis* strains but also of human strains and strains present in contaminated pork (11). In the end, this should provide better insights into the epidemiology of *H. suis* infections worldwide.

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