Short Communication

Genetic relateness of *Haemophilus parasuis* among reference strains and Chinese epidemic isolates

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Abstract

Haemophilus parasuis is the causative agent of Glässer's disease and a commensal coloniser of the porcine upper respiratory tract. Multiple complex factors, including the early weaning of piglets and the management of high health status farms, make it a re-emerging agent, responsible for a recent increase in the prevalence and severity of disease in pigs in China. However, little genetic information is known about Chinese epidemic isolates. In this study, a phylogenetic method for genotyping the *H. parasuis* population with available Chinese epidemic isolates and reference strains from different origins is presented. Phylogenetic analysis confirmed that there are at least two different genotypes in *H. parasuis* population and a unique Chinese lineage with virulence results in the previous epidemics.

Keywords: Haemophilus parasuis; Phylogenetic analysis; Genotype; OMP type; Chinese lineage

Haemophilus parasuis can colonise the porcine upper respiratory tract and also cause severe disease (Oliveira and Pijoan, 2004; Olvera et al., 2007). It is an important bacterial pathogen of pigs in China, being responsible for substantial morbidity, mortality and economic losses (Cai et al., 2005; Li et al., 2009). However, little is known about the genetic relationship between Chinese epidemic isolates, those from other countries and the reference strains. Thus, we performed a phylogenetic analysis of eighteen orthologous genes of strains of *H. parasuis* from different geographical locations and obtained from healthy and disease animals.

Eighteen orthologous genes (*gapA*, *ompP1*, *hscA*, *luxS*, *murG*, *pyrH*, *hlyX*, *ompP2*, *hemN*, *dapA*, *queF*, *lpdA*, *ksgA*, *sodA*, HAPS_1299 (conserved hypothetical protein), *smtA*, *dapB* and *ompP5*) were chosen from orthologous groups according to different functional categories. These were submitted for phylogenomic analysis, along with orthologous genes from 18 complete genomes and two draft genome sequences available for *Pasteurellaceae* (M. Yue et al., unpublished data). MEGA 4.0 was used to create Neighbour-Joining trees with interior branch test (2000 replicates; seed = 80650) (Tamura et al., 2007). The strains and their characteristics and primers used to amplify PCR products for DNA sequencing are detailed in Supplementary Tables S1 and S2, respectively.

Phylogenetically, the strains branched into two main lineages (Fig. 1), indicating that there were two independent genotypes present in the *H. parasuis* population examined. These accorded with results from analysis of *H. parasuis* population by a multilocus sequencing typing (MLST) method used by Olvera et al. (2006b); the only difference was that most of their strains in analysis originated from Europe. Genotype A contained many virulent strains, including a specific Chinese lineage associated with virulence, which also included a cluster (independent sample *t* test, *P* <0.001) containing strains that could not be typed according to the previous serological procedure (F599, F641, F603, F663, F685, F687 and F593) (Cai et al., 2005).

Most of non-typeable strains came from central China (F641, F603, F585 and F593). The majority of Chinese epidemic isolates were composed of serovar 4, serovar 5 and non-typeable strains; the relatively high numbers of non-typeable isolates may reflect the use of serovar 4 and serovar 5 vaccines within China. The reference virulent strains (29755, HS80, HS1080, HS50, HS1079 and HS1081) and another two Chinese clinical isolates (F042 and F093) formed other virulent lineages but there was lack of convergence. However, the American Strain 29755 and HS1080 and Japanese HS80 had a closer genetic relationship with Chinese virulent lineage. The majority of epidemic strains (14/17) in the Chinese lineage (independent sample *t* test, *P* <0.001) indicated that previous *H. parasuis* in China had a genetic specificity different from other virulent lineages.

A cluster containing strains considered to be of lesser virulence (isolated from nasal cavities) formed a unique lineage (independent sample *t* test, *P* <0.001), which included avirulent strains (HS1077, HS1073, HS81 and HS1072), mid-virulent strains (HS83, HS79 and HS1065), two confirmed virulent strains (HS82 and HS1076) and an unclarified strain

HS145. Interesting, the nasal isolates strains HS82 and HS1076 confirmed their virulence in animal challenge, which contradicts the knowledge that healthy nasal isolates were less virulent (Aragon et al., 2009). However, genetic separation between nasal and virulent strains had been observed previously (Olvera et al., 2006a and b).

While genotype A was more robust; genotype B had only two strains (HS1075 and FGXBB), which had a different origin but shared a virulent phenotype. These were also characterised as genotype 2 from analysis of the *H. parasuis* population by the MLST method, which were mostly comprised of clinical disease isolates (24/30) (Olvera et al., 2006b). Analysis of further strains is required to determine the robustness of genotype B.

In addition, we analysed the population structure of the *H. parasuis* strains by comparison of the amino acid sequences of the major outer membrane proteins (OMPs) P1, P2 and P5 in an analogous manner to that described recently (Mullins et al., 2009). The derived phylogenetic tree is shown in Fig. 2. There was a clear separation between OMP type A and B lineages and also a unique Chinese OMP profile within the type A lineage, which also contained genetically closer strains 29755, HS1080 and HS80 (Fig. 1). In a comparison between the two polygenetic trees, the low virulent lineage in Fig. 1 was separated as the two OMP lineages in Fig. 2 (line in green), indicating that strains of nasal origin had more heterogeneity in OMP profiles than their genetic profile. Although immune selection may contribute to a variable OMP profile, a relatively convergent Chinese OMP profile demonstrated the Chinese lineage (independent sample *t* test, P <0.001).

Taken together, the data indicate that there are at least two dramatically different genotypes in the *H. parasuis* population. Phylogenetic analysis of both conserved orthologous genes and OMPs indicate there is a unique Chinese lineage, which is associated with a virulent phenotype. Although the first complete genome sequence of Chinese lineage SH0165 gave valuable genetic information, more work needs to be performed targeting which virulence genes result in disease outcome (Yue et al., 2009).

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary Tables S1 and S2.

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Figure legends

Fig. 1. Neighbour-joining (NJ) tree with interior branch test of 2,000 replicates based on concatenated nucleotide sequences of 18 conserved genes. Thirty-four *H. parasuis* strains could be divided into two genotypes (genotypes A and B). The abundant genotype A contained the Chinese genotype, including the non-typeable cluster and a low virulence cluster (in green). Genotype B had only two strains. *H. influenzae* strain KW20 was used as the reference for the construction of the phylogenetic tree. The NJ tree was produced using MEGA 4.0. The scale bar is in unit of nucleotide substitutions per site. Bootstrap values (%) are indicated at the branch nodes of the phylogenetic trees.

Fig. 2. Neighbour-joining (NJ) tree with interior branch test of 2,000 replicates based on concatenated amino acid sequences of the P1, P2 and P5 proteins. Thirty-four *H. parasuis* strains could be divided into two outer membrane protein (OMP) types or profiles (OMP types A and B). OMP type A contained the Chinese OMP type. Green lines indicate nasal isolates. *H. influenzae* strain KW20 was used as the reference for the construction of the phylogenetic tree. The NJ tree was produced by MEGA 4.0. The scale bar is in units of amino acid substitutions per site. Bootstrap values (%) are indicated at the branch nodes of the phylogenetic trees.

Figure 1

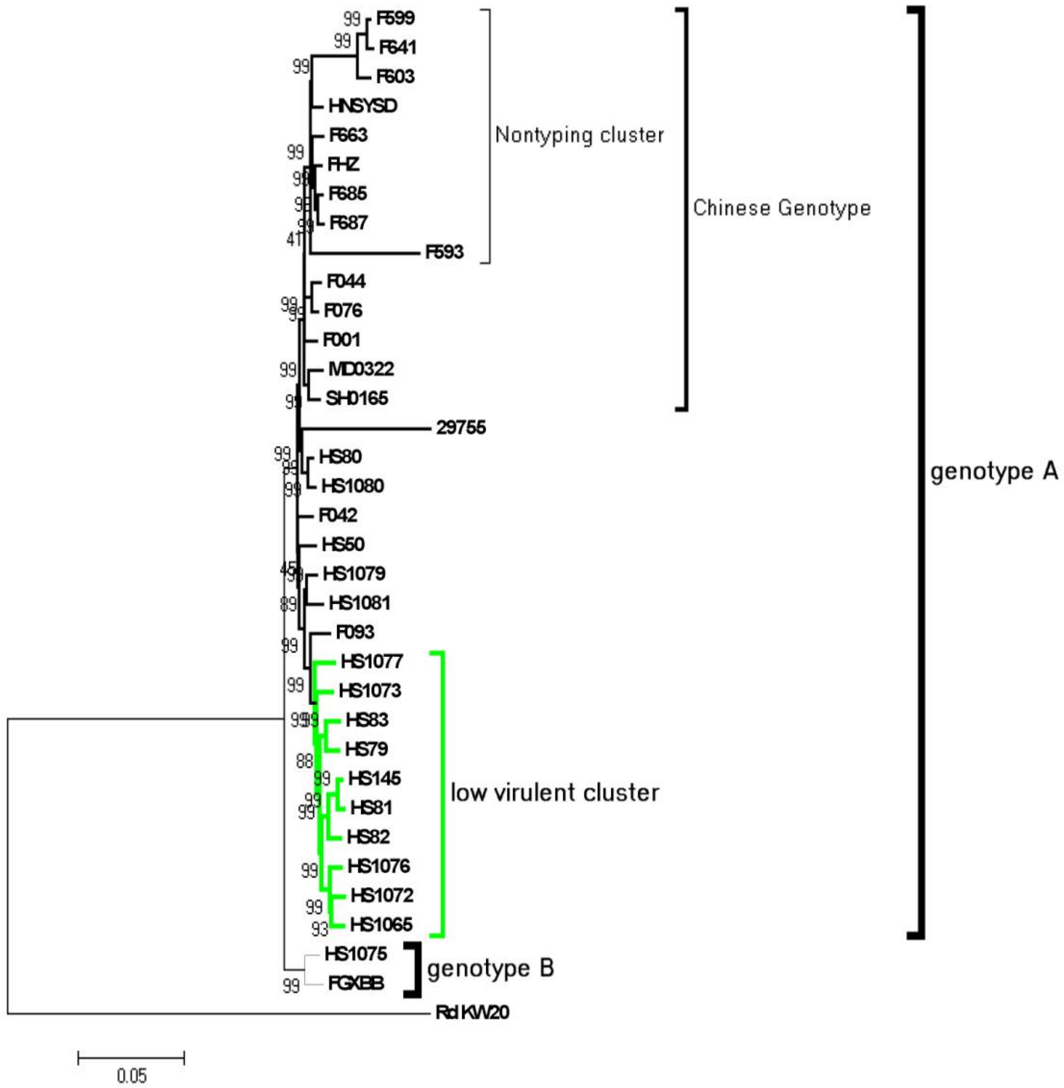
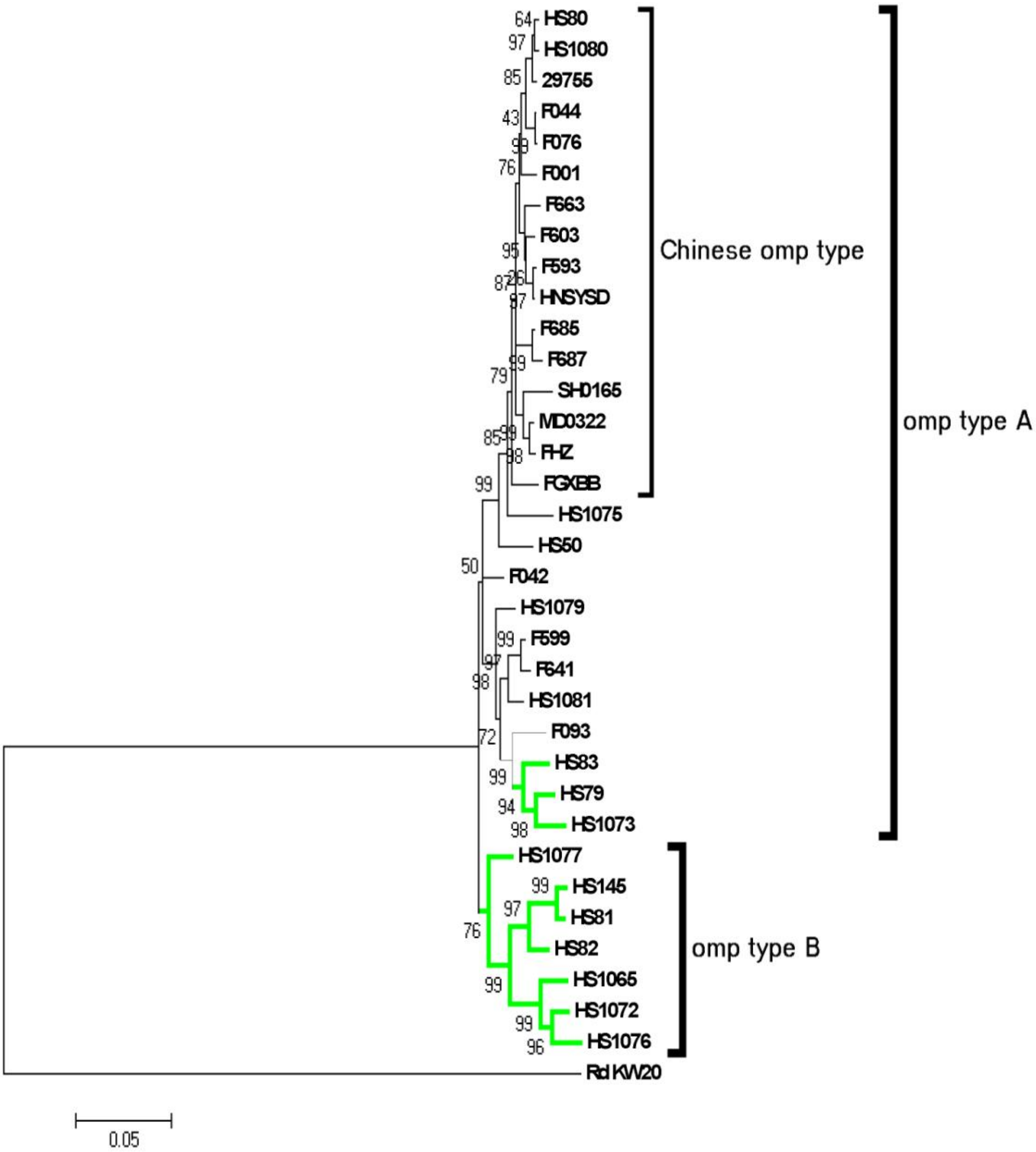

F599
F641
F603
HNSYSD
F663
FHZ
F685
F687
F593
F044
F076
F001
MD0322
SHD165
29755
HS80
HS1080
F042
HS50
HS1079
HS1081
F093
HS1077
HS1073
HS83
HS79
HS145
HS81
HS82
HS1076
HS1072
HS1065
HS1075
FGXBB
Rd KW20
Nontyping cluster
Chinese Genotype
genotype A
low virulent cluster
genotype B
0.05

Figure 2


HS80
HS1080
29755
F044
F076
F001
F663
F603
F593
HNSYSD
F685
F687
SH0165
MD0322
FHZ
FGXBB
HS1075
HS50
F042
HS1079
F599
F641
HS1081
F093
HS83
HS79
HS1073
HS1077
HS145
HS81
HS82
HS1065
HS1072
HS1076
Rd KW20
Chinese omp type
omp type A
omp type B
0.05

Supplementary Table S1

Strains and background information of *Haemophilus parasuis* used in the study.

KRG serovar[a]	Date of receival[b]	Strain	Country of origin[c]	Diagnosis/isolation site	Experimental Infection	Phenotype virulence[d]	Reference
1	12/86	HS82	Japan	Healthy/nose	Glässer’s disease	virulent	Amano et al., 1994
1	2/90	HS145	Australia	ND	ND	ND	Cai et al., 2005
2	12/86	HS83	Japan	Healthy/nose	Healthy	mid-virulent	Nielsen et al., 1993
3	12/86	HS81	Japan	Healthy/nose	Healthy	avirulent	Nielsen et al., 1993
4	12/86	HS79	Japan	Healthy/nose	Subclinical	mid-virulent	Amano et al.., 1994
5	12/86	HS80	Japan	Septicaemia/meningitis	Glässer’s disease	virulent	Amano et al.., 1996
6	9/96	HS1072	Switzerland	Healthy/nose	Healthy	avirulent	Nielsen et al., 1993
7	9/96	HS1073	Switzerland	Healthy/nose	Healthy	avirulent	Nielsen et al., 1993
8	9/96	HS1065	Sweden	ND	Subclinical	mid-virulent	Kielstein et al., 1992
9	11/85	HS50	Sweden	ND	Healthy	avirulent	Kielstein et al., 1992
10	9/96	HS1076	Germany	Healthy/nose	Glässer’s disease	virulent	Kielstein et al., 1992
11	9/96	HS1077	Germany	Pneumonia/trachea	Healthy	avirulent	Kielstein et al., 1992
12	9/96	HS1075	Germany	Polyserositis/lung	Glässer’s disease	virulent	Kielstein et al., 1992
13	9/96	HS1079	USA	ND/lung	Glässer’s disease	virulent	Kielstein et al., 1992
14	9/96	HS1080	USA	ND/joint	Glässer’s disease	virulent	Kielstein et al., 1992
15	9/96	HS1081	USA	Pneumonia/lung	Polyserositis	virulent	Kielstein et al., 1992
5	ND	29755	USA	Pneumonia/lung	Glässer’s disease	virulent	Nielsen et al., 1993
5	4/01	SH0165	Hubei, China	Polyserositis/lung	Glässer’s disease	virulent	Cai et al., 2005
4	8/01	MD0322	Hebei, China	Polyserositis/lung	Glässer’s disease	virulent	Cai et al., 2005
6	/03	F001	Hubei, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
10	/04	F042	Anhui, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
7	/04	F044	Henan, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
5	/04	F076	Fujian, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
2	/04	F093	Hainan, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
NT	/03	F593	Hubei, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005

NT	/04	F599	Shandong, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
NT	/03	F603	Henan, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
NT	/04	F641	Henan, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
NT	/04	F663	Shanghai, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
NT	/04	F685	Hunan, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
NT	/02	F687	Hainan, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
5	/04	FHZ	Zhejiang, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
4	/04	HNSYSD	Hunan, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005
5	/04	FGXBB	Guangxi, China	ND/lung	Glässer’s disease	virulent	Cai et al., 2005

[a] There are 15 serovars according to immunodiffusion using heat-stable antigen extracts and NT was defined as the non-typable strains of *H. parasuis* by serotyping method described by Cai. ND indicated the unknown information.

[b] Date expressed in month/year format.

[c] All the 17 Chinese filed isolates were colleted in 17 distinct farms from 11 provinces representing the Central China (SH0165, MD0322, F001, F044, F593, F603, F641), East China (F042, F076, F599, F663, FHZ), South China (F093, F685, F687, HNSYSD, FGXBB), without disease correlation with available data.

[d] Strains were categorised as the virulent, mid-virulent and avirulent phenotype of *H. parasuis* according to the accompanying reference about experimental infection model. All the 17 Chinese field isolates were provided by the study by Dr. Cai and the detail information about experimental infection with each strains as follows: 9-10 week old weaning piglets (seronegative pigs, 5 for each strain as a group and another 5 for the negative control) were chosen for intraperitobeally challenging with 7×10^{9} CFU, all the infection groups, expect for the negative control, showed clinical syndrome as Glässer’s disease within a week, and 3~5 piglets in each group died in the following weeks (Cai et al., unpublished data).

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Supplementary Table S2

Primers used in this study.

Homologous gene and locus[a]	Primers	Sequence	Expected Length (bp)[b]
gapA	gapA-F	ATGGCAATTAAAATTGGTATTAAC	1020
HAPS_0001	gapA-R	TTAGCCTTTGTAGTTGTAAACGTG	
hscA	hscA-F	ATGTCATTACTTCAAATTGCAGAA	1677
HAPS_0059	hscA-R	TTAATTCCTCACCCGAAAGTAGT	
luxS	luxS-F	ATGCCTTTACTAGATAGCTTTAAAG	510
HAPS_0063	luxS-R	CTATGGATTTAGCAATTTCTCATCT	
murG	MurG-F	ATGACAAAAAAAATTATTGGTAATGG	1056
HAPS_0119	MurG-R	TTACAAACTATTTTCCACAATCACT	
pyrH	pyrH-F	ATGAGCAATCCTATTTATAAACGTA	714
HAPS_0136	pyrH-R	TTAAGCAATCGTCGTACCTTC	
hlyX	hlyX-F	ATGAAAATTGTATCTGATTTTAAAGC	771
HAPS_0167	hlyX-R	TTATAAGTTTGGATTGCAATGGG	
hemN	hemN-F	ATGCAGCCCCCCTTAAGC	1152
HAPS_0238	hemN-R	CTATTCCTTTAAAAAACCTTCCAAC	
dapA	dapA-F	ATGTCAAAACCTCTTTTTTCAGG	888
HAPS_0278	dapA-R	TTAGATTAATTGTGCTTTTTGTAATG	
queF	queF-F	ATGAATTACAATAATGAATGTCTTTCC	840
HAPS_0318	queF-R	TTATTGTCTCACCATTCTTAAATTTT	
lpdA	lpdA-F	ATGAGCCAAGAAATTAAAACACAA	1425
HAPS_0490	lpdA-R	TTATCTTTTCTTCGCTTTTGGAT	
ksgA	ksgA-F	ATGAGTTCAAATTCAAAAAAACATTT	861
HAPS_0737	ksgA-R	TTATTCATCAAACAAGACTAGTTCTTT	
sodA	sodA-F	ATGGCATACACATTACCTGAGTTAG	621
HAPS_0815	sodA-R	TTATGCTTGGGATTCAAAACGT	
HAPS_1299	HAPS1299-F	ATGATTTACAGTATGACCGCTTTC	864
HAPS_1299	HAPS1299-R	CTACTCCAAATTCTGAATTTGCTC	
smtA	smtA-F	ATGGGAACAAAGAATTGTTCCTA	789
HAPS_1395	smtA-R	CTAATGAGATAGATATATATCAAACCGA	
dapB	dapB-F	ATGACATTAAAAATTGGCGTTGT	813
HAPS_2274	dapB-R	TTATAAATTATTTAAATCCAACACATCG	
ompP1	ompP1-F	ATGAATAAATTTACTAAAACAGCACTT	1290
HAPS_0037	ompP1-R	TTAGAATTTGTAGTTTACGTTTAAGC	
ompP2	ompP2-F	ATGAAAAAAACACTAGTAGCATTAGC	1092
HAPS_0164	ompP2-R	TTACCATAATACACGTAAACCAACA	
ompP5	ompP5-F	ATGAAAAAATCTTTAATTGCATTAGC	1116
HAPS_2298	ompP5-R	TTACATAGAAACTTCTTTTGAACCTT	

[a] Gene name was assigned according to the *H. parasuis* genome SH0165. The homologous gene locus number is given below each gene name.

[b] Expected length of the PCR amplification product according to the homologous gene in SH0165 genome.