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Research paper

Multilocus sequence typing and virulence analysis of *Haemophilus parasuis* strains isolated in five provinces of China

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ABSTRACT

Haemophilus parasuis is the etiological agent of Glässers disease, which causes high morbidity and mortality in swine herds. Although *H. parasuis* strains can be classified into 15 serovars with the Kielstein–Rapp-Gabrielson serotyping scheme, a large number of isolates cannot be classified and have been designated 'nontypeable' strains. In this study, multilocus sequence typing (MLST) of *H. parasuis* was used to analyze 48 *H. parasuis* field strains isolated in China and two strains from Australia. Twenty-six new alleles and 29 new sequence types (STs) were detected, enriching the *H. parasuis* MLST databases. A BURST analysis indicated that *H. parasuis* lacks stable population structure and is highly heterogeneous, and that there is no association between STs and geographic area. When an UPGMA dendrogram was constructed, two major clades, clade A and clade B, were defined. Animal experiments, in which guinea pigs were challenged intraperitoneally with the bacterial isolates, supported the hypothesis that the *H. parasuis* STs in clade A are generally avirulent or weakly virulent, whereas the STs in clade B tend to be virulent.

1. Introduction

Haemophilus parasuis is a Gram-negative bacterium of the family Pasteurellaceae (Biberstein et al., 1969). It is the etiological agent of Glässers disease, causing symptoms of polyarthritis, fibrinous polyserositis, and meningitis (Little, 1970). *Haemophilus parasuis* strains can be classified into 15 serotypes based on immunodiffusion using heat-stable antigen extracts, but a large number of *H. parasuis* isolates cannot be classified with this method, and are designated 'nontypeable' (NT) strains (Del Río et al., 2003; Kielstein et al., 1992; Morozumi et al., 1986; Tadjine et al., 2004). This limits the utility of serotyping in epidemiological surveys and in the analysis of population structures (Mullins et al., 2013). To resolve this problem, fingerprinting methods, such as multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and enterobacterial repetitive intergenic consensus sequences, have been used to differentiate *H. parasuis* strains. These methods are more discriminatory than serotyping, but their results are not subjective and cannot be readily compared among laboratories on a global scale (Olvera et al., 2007a, b).

Multilocus sequence typing (MLST) is a genotyping method based on sequence data from 6 to 10 housekeeping genes (Maiden et al., 1988). The mutations in strains are analyzed with PCR amplification and sequencing to identify the slowly accumulating genetic variation in housekeeping genes that reflects the evolutionary relationships between strains. Thus, all isolates can be definitively classified and the results are readily comparable among laboratories on a global scale (Andersson et al., 2012; Elberse et al., 2011; Mullins et al., 2013). MLST has been used successfully to analyze bacteria such as *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Escherichia coli*, and others (Andersson et al., 2012; Elberse et al., 2011; Larsen et al., 2012; Liang et al., 2014). In *H. parasuis* research, Olvera et al. (2006) first used the MLST method to analyze *H. parasuis* strains and Mullins et al. (2013) optimized the MLST typing scheme so that it more accurately defines the population structures and genetic diversity of *H. parasuis*.

In this study, we analyzed 48 *H. parasuis* field strains from China and two strains from Australia with the MLST method, to extend our understanding of the evolution and population genetics of these *H. parasuis* strains. Animal experiments were also performed to compare the pathogenicity of some strains, to analyze the correlation between the *H. parasuis* clades and the virulence of strains.

2. Materials and methods

2.1. Bacterial strains

Sixty-five *H. parasuis* strains were used in this study, including 15 recognized serovar reference strains and 50 field strains (Table 1).

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Table 1
List of *Haemophilus parasuis* strains with their correspondent sequence type (ST), allelic profile, organ, Disease and region of isolation.

Serotype	Strain	ST	atpD	infB	mdh	rpoB	6pgd	g3pd	frdB	Organ	Disease	Region	Date
7	174[a]	4	2	1	8	6	8	3	8	Nose	Healthy	Switzerland	UK
13	17,975[a]	5	1	1	1	1	1	1	1	Lung	UK	USA	UK
15	15,995[a]	15	6	10	3	1	3	3	2	Lung	Pneumonia	USA	UK
8	C5[a]	17	4	5	14	4	13	3	14	UK	UK	Sweden	UK
10	H367[a]	18	5	9	5	1	10	1	10	UK	UK	Germany	UK
12	YZ-12	19	3	1	9	1	3	1	2	UK	UK	Jiangsu	2014
12	H425[a]	19	3	1	9	1	3	1	2	Lung	Polyserositis	Germany	UK
11	H465[a]	20	2	1	5	5	10	5	11	Lung	Pneumonia	Germany	UK
1	NO.4[a]	23	2	4	4	2	9	3	9	Nose	Healthy	Japan	UK
5	H5R	24	1	8	7	1	1	1	3	Lung	Pneumonia	Gansu	2008
5	H08	24	1	8	7	1	1	1	3	Brain	Meningitis	Guangdong	2007
5	YZ-5	24	1	8	7	1	1	1	3	UK	UK	Jiangsu	2014
5	Nagasaki[a]	24	1	8	7	1	1	1	3	Meninges	Septicaemia	Japan	UK
9	D74[a]	25	4	5	5	4	5	3	5	UK	UK	Sweden	UK
3	SW114[a]	26	4	6	6	4	6	3	6	Nose	Healthy	Japan	UK
4	YZ-4	27	2	7	4	5	7	4	7	UK	UK	Jiangsu	2014
4	30	27	2	7	4	5	7	4	7	UK	UK	Jiangsu	2007
4	SW124[a]	27	2	7	4	5	7	4	7	Nose	Healthy	Japan	1986
NT	JX5	27	2	7	4	5	7	4	7	UK	UK	Jiangxi	2007
2	SW140[a]	28	2	14	15	5	2	3	15	Nose	Healthy	Japan	UK
14	22,113[a]	113	3	29	1	1	1	1	20	Joint	Septicaemia	USA	UK
6	131[a]	114	20	5	28	13	35	3	6	Nose	Healthy	Switzerland	UK
5	H9	158	10	10	7	1	19	1	20	Lung	Pneumonia	Guangdong	2007
7	H19	159	7	2	6	3	27	3	9	Heart blood	Polyserositis	Guangdong	2007
7	H20	159	7	2	6	3	27	3	9	Heart blood	Polyserositis	Guangdong	2007
NT	K3	160	4	18	17	4	15	3	6	Lung	Pneumonia	Jiangsu	2009
10	H38	160	4	18	17	4	15	3	6	Lung	Polyserositis	Guangdong	2008
10	H39	160	4	18	17	4	15	3	6	Lymph node	Polyserositis	Guangdong	2008
7	16	161	4	4	6	3	27	3	21	Lung	Pneumonia	Jiangsu	2008
7	YT	161	4	4	6	3	27	3	21	Lung	Pneumonia	Jiangsu	2008
7	HPS4	161	4	4	6	3	27	3	21	Lung	Pneumonia	Heilongjiang	2009
5	H17	162	10	17	7	1	19	1	17	Lung	Pneumonia	Guangdong	2007
5	H14	162	10	17	7	1	19	1	17	Lung	Pneumonia	Guangdong	2007
7	HS197	163	4	34	30	17	12	3	31	UK	UK	Australia	UK
4	H25	164	27	9	4	1	3	1	3	Inguinal effusion	Polyserositis	Guangdong	2008
NT	H24	164	27	9	4	1	3	1	3	Lung	Pneumonia	Guangdong	2008
NT	H23	164	27	9	4	1	3	1	3	Lung	Pneumonia	Guangdong	2008
NT	211/212	165	2	1	4	5	44	3	2	Lung	Pneumonia	Jiangsu	2008
NT	QIXIAN	166	2	9	1	6	17	3	38	Lung	Pneumonia	Shanghai	2007
5	H04	167	25	2	32	1	3	17	36	UK	UK	Guangdong	2007
5	3031	167	25	2	32	1	3	17	36	UK	UK	Jiangsu	2007
NT	3032	167	25	2	32	1	3	17	36	UK	UK	Jiangsu	2007
14	H12	168	26	40	33	1	43	1	2	Lymph node	Polyserositis	Guangdong	2007
NT	H13	168	26	40	33	1	43	1	2	Lung	Polyserositis	Guangdong	2007
4	H40	169	24	39	36	1	45	1	36	Lung	Pneumonia	Guangdong	2008
13	YZ-13	170	5	1	6	19	46	1	1	UK	UK	Jiangsu	2014
NT	H26	171	24	2	37	1	47	1	40	Heart blood	Polyserositis	Guangdong	2008
1	HS145	172	7	9	4	6	48	3	2	UK	UK	Australia	UK
NT	ZH3	173	21	9	4	5	4	18	2	Lung	UK	Jiangsu	2008
12	HPS7	174	24	11	35	1	45	1	37	Lung	Pneumonia	Heilongjiang	2010
5	H45	175	28	10	34	1	43	3	39	Pericardial effusion	Polyserositis	Guangdong	2008
NT	H44	176	26	39	34	1	43	1	2	Lung	Polyserositis	Guangdong	2008
5	H10	177	5	10	33	1	10	1	2	Brain	Meningitis	Guangdong	2007
1	HPS6	178	2	2	22	6	12	3	23	Lung	Pneumonia	Heilongjiang	2009
7	1117	179	7	4	6	3	27	3	38	Lung	Polyserositis	Qinghai	2008
12	H27	180	25	4	4	1	3	1	9	Joint fluid	Polyserositis	Guangdong	2008
12	H31	180	25	4	4	1	3	1	9	Lung	Pneumonia	Guangdong	2008
15	H35	181	26	11	12	1	43	3	38	Heart blood	Polyserositis	Guangdong	2008
14	H36	181	26	11	12	1	43	3	38	Joint fluid	Polyserositis	Guangdong	2008
NT	H33	181	26	11	12	1	43	3	38	Heart blood	Polyserositis	Guangdong	2008
5	H46	182	26	10	34	1	43	3	39	Pericardial effusion	Polyserositis	Guangdong	2008
5	H15	183	26	17	7	1	19	1	17	Heart blood	Polyserositis	Guangdong	2007
NT	H47	184	24	11	36	1	45	1	37	Brain	Polyserositis	Guangdong	2008
NT	H49	185	4	5	19	13	13	3	21	UK	UK	Guangdong	2008
NT	H21	186	26	11	12	1	43	1	3	Lung	Pneumonia	Guangdong	2007

It was sorted in ascending order of ST numbers. NT means nontypeable; UK means unknown.
[a] Reference strains.

Forty-eight of the 50 field strains collected in five provinces in China were isolated from the lungs, brain, or hydropericardium and other systemic sites of sick pigs. Two of the 50 field strains were from Australia, and were kindly provided by Dr. P. J. Blackall of the Animal Research Institute (Queensland, Australia). They were classified with the agar gel diffusion method (Kielstein et al., 1992), and antisera were kindly provided by Prof. Huanchun Chen of Huazhong Agricultural University (Wuhan, China).

Haemophilus parasuis strains were cultured in trypticase soy broth (TSB) or on trypticase soy agar (TSA) (BD Westminster, CO, USA)