Microbial Pathogenesis 77 (2014) 17–23

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Identification of putative virulence-associated genes among *Haemophilus parasuis* strains and the virulence difference of different serovars

Jiang Yu [a, b, c, 1], Jiaqiang Wu [a, b, 1], Yuyu Zhang [a, b], Yijun Du [a, b], Jun Peng [a, b], Lei Chen [a, b], Wenbo Sun [a, b], Xiaoyan Cong [a, b], Shaojian Xu [a, b], Jianli Shi [a, b], Jun Li [a, b], Baohua Huang [a, b], Xiwang Zhu [a, b], Jinbao Wang [a, b, c, *]

[a] Shandong Academy of Agricultural Sciences, Jinan 250100, China
[b] Shandong Key Lab of Animal Disease Control and Breeding, Jinan 250100, China
[c] Shandong Normal University, Jinan 250014, China

ARTICLE INFO

Article history:
Received 4 May 2014
Received in revised form
29 September 2014
Accepted 1 October 2014
Available online 2 October 2014

Keywords:
Haemophilus parasuis
Serovars
Virulence evaluation
Virulence genes

ABSTRACT

This study was aimed at determining virulence-associated genes among *Haemophilus parasuis* (*H. parasuis*) strains, and supplying for the Kielstein–Rapp–Gabrielson serotyping scheme. The subtractive fragments, obtained through suppression subtractive hybridization and reverse Southern blot hybridization, were found to encode genes representative of 7 different functions. PCR was used to investigate the distribution of these fragments in *H. parasuis* strains isolated from different infection sites in pigs. Mice challenge was then used to analyze the correlationship between subtractive fragments, infection sites and bacterial virulence. Eight weeks old female BALB/c mice (10 mice/group) were inoculated intraperitoneally with 3.0×10^9 CFU suspension (0.5 ml/mouse) of *H. parasuis* strains in PBS. Results indicated that *H. parasuis* possessed varied virulence even among the same serovar strains. Transcription units hsdR, hsdS, gpT and ompP2, identified from the subtractive fragments, were uniformly expressed in highly virulent strains, while absent in weakly virulent strains, and demonstrated variable degrees of expression in moderately virulent strains. Moreover, *H. parasuis* strains, isolated from pericardium and heart blood, were all highly virulent strains, while from nasal cavity and joint were moderately or weakly virulent strains. This study indicated that fragments hsdR, hsdS, gpT and ompP2 were associated with the virulence of *H. parasuis*. The virulence of *H. parasuis* strains isolated from different infection sites was different. The current research provides a new reference for determining bacterial virulence in different *H. parasuis* strains.

1. Introduction

Haemophilus parasuis (*H. parasuis*), a ubiquitous bacterium in the upper respiratory tract of conventional pigs, is the causative agent of Glässer's disease. Several *H. parasuis* strains are virulent, consequently causing diseases under certain conditions [1,12,17,19]. This bacteria can be isolated from the nasal passages of asymptomatic swine, as well as those with septicemia and pneumonia without polyserositis [15,35].

During the last few years, this disease has been emerging as one of the main causes of nursery mortality in modern swine husbandry, leading to significant economic losses worldwide [7,22]. To date, the classification system most widely used for *H. parasuis* has been based on serotyping [25]. This system is based on the use of a battery of hyperimmune antisera, which precipitate in gel diffusion (GD) tests. A total of 15 different serovars of *H. parasuis* have been described, largely differing in their virulence from ranging highly virulent to nonvirulent [9,18,29]. However, the Kielstein–Rapp–Gabrielson serotyping scheme has been established in early the 1990s, and the virulence of *H. parasuis* strains may have changed in the past 20 years. Thus, the new methods determining the virulence of *H. parasuis* strains should be found, which could supply for the Kielstein-Rapp-Gabrielson serotyping scheme.

* Corresponding author. Shandong Key Lab of Animal Disease Control and Breeding, Shandong Academy of Agricultural Sciences, No. 8, Sangyuan Rd., Jinan 250100, China.
E-mail address: jinbaowang@aliyun.com (J. Wang).
[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.micpath.2014.10.001
0882-4010/

Several molecular strategies including microarray analysis [14], differential display reverse transcription-polymerase chain reaction (DDRT-PCR) [7], selective capture of transcribed sequences (SCOTS) [8] and sequencing [5] have been used to identify virulence-associated genes in bacteria. Certain significant virulence markers, such as outer membrane protein P5 (ompP5) and cytolethal distending toxin (CDT), have been identified [36,37]. However, more virulence-associated factors and virulence markers for the varied degrees of virulence observed in *H. parasuis*, still need to be authenticated at present.

Of all *H. parasuis* strains, LC (serovar 1), has a certain degree of cross-reaction with serovars 4, 5, 12 and 13 by GD test. Moreover, strains ZQ (serovar 4) and LZ (serovar 5), were the most prevalent serovars in China. In the previous study, *H. parasuis* strains LC, ZQ, and LZ were used to infect the conventional pigs to confirm the virulence of the strains. Results showed that the pathogenicity of ZQ was mild (data not showed), while LC and LZ could cause serious polyserositis and arthritis [33]. Thus, strain LC, which has a cross-reaction with serovars 4, 5, 12 and 13, was selected to screen subtractive genes differing from strain ZQ.

In the current study, genetic differences were conducted to compare between *H. parasuis* highly virulent strain LC (serovar 1) and weakly virulent strain ZQ (serovar 4) using SSH and reverse Southern blot hybridization. Transcription units, hsdR, hsdS, gpT and ompP2, were confirmed to be virulence-associated genes by PCR and mice challenge. We compared the degree of pathogenicity of subtractive fragments from different *H. parasuis* strains in mice. Our findings would be useful in distinguishing the virulence of different *H. parasuis*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The different serovar *H. parasuis* strains used in this study had been isolated from pig herds of China. *H. parasuis* strains were cultivated in Tryptic Soy Agar (TSA, Difco Laboratories, Detroit, MI) containing 10 μg/ml nicotinamide adenine dinucleotide (NAD) and 5% bovine serum at 37 °C in a 5% CO_2 enriched atmosphere.

2.2. Serotyping

Reference strains of *H. parasuis* serovars 1–15 were stored at Shandong Key Lab of Animal Disease Control and Breeding. Hyperimmune antisera to *H. parasuis* were produced in rabbits as previously described [21]. All the isolates of *H. parasuis* were serotyped by gel diffusion (GD) test. The autoclaved antigen for the GD test was prepared as described by [29].

2.3. Genome extraction of all the H. parasuis strains

Bacterial cultures were harvested from TSA medium and suspended into 100 ml of PBS (pH 7.0). The suspension was boiled for 10 min, followed by cooling on ice for 5 min. After melting, the suspension was centrifuged at 12,000× *g* for 5 min and the supernatant was collected and stored at −20 °C until use.

2.4. Construction and screening of SSH library

Subtractive hybridizations between *H. parasuis* strains, LC (highly virulent) and ZQ (weakly virulent), were performed using a PCR-Select™ Bacterial Genome Subtractive Kit as recommended by the manual operation (Clontech Laboratories, Inc., CA, USA). Initially genomic DNAs of LC and ZQ strains, were digested with *RsaI* (New England Biolabs, Mississauga, Ontario, Canada).

Genomic DNA from the *RsaI*-digested LC strain was subdivided into two equal quantities, and was used as the tester DNA for two separate hybridizations. Genomic DNA from the *RsaI*-digested ZQ strain was used as the driver DNA for both hybridizations.

Two rounds of PCR were performed and the secondary PCR products were cloned into the pMD19-T vector (TAKARA, Dalian, China) using the Advantage PCR Cloning Kit (Clontech Laboratories, Inc., CA, USA.) and transformed into *Escherchia coli* DH5α cells (TransGen, Beijing, China). The transformants were plated on Luria–Bertrani medium (LB) agar with 100 μg/ml ampicillin. Individual colonies were picked and grown in LB broth supplemented with ampicillin overnight at 37 °C. Plasmid DNA was isolated using the prep Mini Plasmid Kit (TIANGEN, Dalian, China). Those plasmids containing inserts were screened by PCR, followed by the separation of the fragments on 1% agarose gels.

2.5. Reverse southern blot hybridization of subtractive products

Two probes were prepared using 2 μg of RsaI-digested genomic DNA of LC and ZQ strains, respectively. The probes were separately labeled using digoxigenin (DIG)-High Prime (Roche Applied Science, Indianapolis, USA).

PCR products of all clones from the SSH library were transferred to a positively charged Nylon membrane (Amersham Pharmacia, USA) for 2 h at 80 °C. Duplicate blots were separately hybridized with LC or ZQ genomic DNA probes for 16 h at 42 °C. Washing and detection were carried out in accordance with the instructions (DIG DNA Labeling and Detection Kit, Roche, Germany).

2.6. Sequence analysis and PCR amplification

The screened plasmid DNA was sequenced. Sequence analysis was carried out using the BLAST from the National Center for Biotechnology Information (NCBI).

PCR primers (Table 1) were designed to amplify the subtractive fragments from 39 *H. parasuis* strains. The results of PCR were used

Table 1
Primers used in this work.

Primers	Sequences (5′–3′)	G + C content (%)	Annealing temperature (°C)	Fragment size (bp)
hsdR-F	ATGACTATTGAAACAACGCCTATCA	36	60.3	544
hsdR-R	GAACTTGGTTAAAGGCTTCC	45	53.7	
hsdS-F	ATGATAGAAAGTCGTTTTATTGACC	24	55.3	819
hsdS-R	GGGTTTGGCATTATCTGTCC	45	55.5	
thiD-F	TTAAGCCGATATTGCCCAATGATTT	36	65.4	687
thiD-R	ACAGCAGTTACCGCTCAAAA	45	56.1	
thiE-F	TCAATATGTTGTCTTTAATTTCTGC	28	56.2	338
thiE-R	TTCAATTAACGCAAGGCAAG	40	56.9	
thiM-F	CTAGATAATCCATTCTGCTTTCATT	32	56.8	743
thiM-R	ATTTGGTTTCAGCGCATTTT	35	57.7	
int-F	CTATGCAACTAATCTTAATCGTGGT	36	57.5	1055
int-R	TGTCGATCTACAGACGAAAA	40	51.2	
msrA-F	ATGGTTACGCATACAGTCATTATCA	36	58.8	660
msrA-R	ACAGTGGTTACCGCTTGG	55.6	53.6	
gpT-F	GTATGTCTGAAGCATTATTGAAAAC	32	55.6	1480
gpT-R	TATGACATCGCCTCCACCAG	55	58.9	
prtR-F	ACCGCATAAACCTCACCATC	50	57.3	519
prtR-R	ATGAGTACATTAGGCGAACGAATTG	40	62.1	
gntP-F	ATGTTTGGGCTACCGATTCCTATTA	40	62.8	1216
gntP-R	AACCCATTGTTGCTGCCTGT	50	60.2	
gst-F	ATGAAACTTTATTATCTCCCAGGCG	40	62.6	584
gst-R	TCTACACCTTTATCCGCTTC	45	52.3	
serC-F	ATGACACAAGTTTATAATTTTTGTG	28	53.6	1009
serC-R	AGATTGATGCACGCATACCG	50	59.3	
ompP2-F	ATGAAAAAAACACTAGTAGCA	28.6	41.7	1092
ompP2-R	TTACCATAATACACGTAAACC	33	41.9	