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Full Length Research Paper

Phylogenetic and pathogenetic analysis of *Streptococcus suis* serotype 7 strain HW07 isolated from diseased pig in China

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A recent isolate of *Streptococcus suis* serotype 7 (*SS7*), named HW07, was isolated from brains of pigs suspected with *Streptococcus suis (S. suis)* infection. The sequence types (ST) of this isolate was determined by Multilocus sequence typing (MLST) method. In order to evaluate pathogenesis of HW07 isolate, zebrafish and pigs were inoculated with HW07 strain by peritoneal cavity and vein. The results demonstrate that the 50% lethal dose (LD_{50}) of HW07 isolate was 1.25×10^5 colony forming units (CFU)/fish and could induce pig disease, the ST number of HW07 isolate is a novel ST-ST335. Molecular and phylogenetic analysis for the *recA* gene of HW07 isolate showed that it joins to the I group cluster of *S. suis* strains, the predominant genotype in China.

Key words: *Streptococcus suis serotype 7*, multilocus sequence typing, the sequence type, phylogenetic analysis.

INTRODUCTION

Streptococcus suis is an important pathogen associated with a variety of pig diseases, including meningitis, septicaemia, arthritis, endocarditis and pneumonia and can cause severe zoonotic infection of humans. So far, 35 capsular serotypes of S. suis have been described (Perch et al., 1983), serotype 2 strains are considered as the most virulent and often isolated from diseased pigs. However, epidemiological studies in our lab have indicated that the prevalence of SS7 strains from diseased pigs in China has significantly increased during the past 3 years (Wang et al., 2012). Therefore, in this study, we researched in SS7 strains HW07 isolated from brains of diseased pigs with classic clinical meningitis and arthritis symptoms of *S. suis* infection, which had resulted in 30% death rate of pigs in farm.

Pathogenicity can vary substantially both within and among serotypes, and not all isolates of the same serotype cause the same disease (Staats et al., 1997). Previous studies have indicated that *S. suis* is a genetically diverse species (Hampson et al., 1993). In order to determine the genotype or sequence of the HW07 isolates, MLST method was used in this study. MLST is a highly discriminatory and unambiguous method of characterizing bacterial isolates that has now been successfully employed in the characterization of several species (Enright et al., 2001). MLST was used to investigate the genotype of *S. suis* as early as in 2002 (King et al., 2002). Strains that have the same ST number are identical at all of the sequenced loci and are considered to be members of the same clone, which means they have a recent common ancestor. Up to August 2012, 665 *S. suis* strains have been recorded in the *S. suis* MLST Database and classified into 334 STs which belong to 21 ST complexes.

The recA protein is a multifunctional enzyme that plays a role in homologous recombination, DNA repair and induction of the SOS response (Selbitschka et al., 1991). RecA sequencing is an adequate method to discriminate

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Table 1. The Primers for virulence-associated gene

Primer	Primer sequence(5'-3')	Gene	Protein	GenBank accession number	Length of PCR plroduct (bp)
Gdh	GAGCTCTTCTCTACACTT	*gdh*	Glutamate dehydrogenase	AY853916	1257
	TTATACCAAACCTTGGGC				
Mrp	ATCAGAATCACCACTTTTGG	*mrp*	Muramidase-released protein	X64450 [31]	885
	TCATACCCAGTAAATACACG				
Ef	CGCAGACAACGAAAGATTGA	*epf*	Extracellular factor	X71881 [31]	744
	AAGAATGTCTTTGGCGATGG				
Sly	GCTTTATTGCGTGCTGAC	*sly*	Suilysin	Z36907 [31]	1097
	CTGTTCTCCACCACTCCC				

among meat staphylococci, *S. xylosus* and *S. equorum*. The objective of the current study was to determine the genotype and pathogenesis of HW07 isolates and to investigate if it was the predominant *S. suis* strain in China by sequence and phylogenetic analysis of the recA gene.

MATERIALS AND METHODS

Strains

The brain sample was collected from the infected pig and the bacteria was isolated using a method previously described (Buddle et al., 1981). The *S. suis* suspected colonies. The Strain 8074 is the international reference *SS7* that was stored in our research institute.

Genomic DNA extraction

The isolate chromosomal DNA was extracted using a method previously described (Vaquero et al., 2004) . Bacteria grow in THB at 37°C before chromosomal DNA was extracted from overnight cultures using TIANamp Bacteria DNA Kit (TIANGEN, China) according to the manufacturer's instructions.

Strain identification

The *S. suis* suspected colonies were characterized by morphological, biochemical (API 20 STREP, biomerieux, France) and based PCR assay. The presence of *S. suis* was confirmed by PCR with *S. suis gdh* gene primers (Okwumabua et al., 2003): FP: 5'-GCAGCGTATTCTGTCAAACG-3 RP: 5'-CCATGGACAGATAAAGATGG-3', the expected fragment was 688 bp. Serotype 7 was confirmed by PCR with *S. suis cps7H* gene primers (Okwumabua et al., 2003): FP: 5'-AATGCCCTCGTGGAATACAG-3', RP: 5'-TCCTGACACCAGGACACGTA-3', the expected fragment was 378 bp. The identified *SS7* clones were stored at -40°C.

The virulence-associated genotype of HW07 isolate

Based on the presence of virulence-associate gene to characterize the *SS7* strain, primers based on the following gene are listed in Table 1: Glutamate dehydrogenase (*gdh*), muramidase-released protein (*mrp*), extracellular factor (*epf*), and suilysin (*sly*) (Silva et al., 2006). PCR was performed to characterize the virulence-associated genotype of HW07 isolate using the four virulence-associated gene primers.

MLST and phylogenetic analysis

MLST was performed according to King et al. (2002). PCR amplification of the 7 housekeeping genes include aroA, cpn60, dpr, gki, recA, and thrA was done with the primers shown in Table 2. The amplified segments were sequenced at Huada gene Biotechnological Co. Ltd (Beijing, China).

MLST alleles and ST number of HW07 isolate was analysed in *S. suis* MLST Database (http://ssuis.mlst.net). eBURST software (Feil et al., 2004) was used to identify the phylogenetic position of strain and display the overall structure of the population.

Phylogenetic analysis for recA gene

The partial *recA* gene nucleotide of HW07 isolate was sequenced and was sent to GenBank, in which the accession numbers for *recA* was JX236275. Sequence similarity searches in the GenBank databases were carried out using Basic local alignment search tool (BLAST), and aligned with the corresponding sequences of *S. suis* strains using the Clustal W program in MegAlign of Lasergene 7.0 software (DNASTAR Inc. Madison, WI, USA). Then, the molecular and the phylogenetic analyses of *recA* gene was conducted by Molecular Evolutionary Genetics Analysis (MEGA) version 5.05 (Tamura et al., 2011). The percentage of bootstrap confidence levels for internal branches, as defined by the MEGA program, was calculated from 1000 random resamplings.

Experimental animals

10-week-old inbred line zebrafish, which were purchased from National Zebrafish Resources of China (Shanghai, China) and raised in solated fish bowls, were used to check the virulence of HW07 isolate. Six 1-month-old Specific pathogen Free (SPF) pigs, whose serology is

Table 2. Primers used for amplification and sequencing of the seven loci in the *S. suis* MLST scheme.

Gene name	Primer sequence	Annealing Tm (℃)	Length of PCR(bp)
aroA-FP	TTCCATGTGCTTGAGTCGCTA	55	482
aroA-RP	ACGTGACCTACCTCCGTTGAC		
cpn-FP	TTGAAAAACGTRACKGCAGGTGC	52	466
cpn-RP	ACGTTGAAIGTACCACGAATC		
dpr-FP	CGTCTTTCAGCCCGCGTCCA	50	434
dpr-RP	GACCAAGTTCTGCCTGCAGC		
gki-FP	GGAGCCTATAACCTCAACTGG	55	480
gki-RP	AAGAACGATGTAGGCAGGATT		
mutS-FP	CGCAGAGCAGATGGAAGATCC	50	526
mutS-RP	CCCATAGCTGTTTTGGTTTCATC		
recA-FP	TATGATGAGTCAGGCCATG	50	398
recA-RP	CGCTTAGCATTTTCAGAACC		
thrA-FP	GATTCAGAACGTCGCTTTGT	52	523
thrA-RP	AAGTTTTCATAGAGGTCAGC		

negative to *S. suis*, were purchased from Dongsheng pig form (Harbin, China). All animal work and experimental procedures were conducted with an approval of Institutional Animal Care and Use Committee of Heilongjiang, China.

Pathogenicity test of HW07 isolate to zebrafish

The isolated bacteria were harvested from liquid cultures by centrifugation at 5,000 × g for 5 min prior to inoculation in the zebrafish, and were resuspended in phosphate-buffered saline (PBS, pH 7.4). Strains of HW07 and 8074 were tested at 5 different doses from 5×10^7 to 5×10^3 CFU/fish, ten fish per dose. The zebrafish were injected through peritoneal cavity, and the control fish were injected with PBS. The infected zebrafish were monitored for 1 week. The test was repeated in triplicate and yielded reproducible results. The results were averaged and used to calculate the LD_{50} by the method of Reed and Muench (Reed and Muench, 1938).

Pathogenicity test of HW07 isolate to piglet

To evaluate the pathogenicity of the HW07 isolate, four-week-old piglets (3 piglets) were challenged with the HW07 isolate (dose of 2×10^8 CFU/piglet), and the control piglets (3 piglets) were challenged with PBS. Clinical signs and rectal temperatures of the piglets were daily recorded until the piglets were euthanized with Nembutal at the end of the experiment. All the tissue samples of infected piglets were obtained. Bacteria were re-isolated from various tissues.

RESULTS

Morphology and characteristics of recently SS7 isolate

A wild-type SS7 isolate, named HW07, was isolated from the brain of the infected pig, which was Gram positive chain shape coccobacteria by microscope observation.

The results of biochemical tests (6.5% high salt gravy/ Raffinose/ lactose/ sorbose/ mannose/ salicin/ serum dahlin/ hippurate/ Esculin/ mushroom sugar: positive/ positive/ positive/ negtive/ positive/ positive/ negtive/ negtive/ positive/ positive) were consistent with the biochemical character of *S. suis*. The PCR result for isolate was gdh+/cps7H+.

The virulence-associated genotype of HW07 isolate

In order to determine the virulence-associated gene of isolate, four genes (gdh, mrp, epf and sly) were examined by PCR. As shown in Figure 1, the virulence-associated gene of isolate was gdh+/mrp+/epf--/sly-.

MLST and phylogenetic analysis

Partial sequences of 7 housekeeping genes for isolate revealed a low genetic variation, yielding one novel ST number-ST335 whose allelic profiles was 8, 30, 5, 34, 9, 3, 25. The closest matches are ST129 (8, 30, 5, 34, 58, 3, 25), ST83 (8, 30, 5, 34, 39, 3, 25) and ST29 (8, 30, 5, 34, 30, 3, 25). From the above results we can conclude that five isolates come from a clone. The phylogenetic position of the isolate in the *S. suis* database and the overall structure of the population are displayed in Figure 2.

Phylogenic and sequencing analysis of recA gene within HW07 isolate

Phylogenetic analysis of recA gene was conducted in HW07 isolate and 23 *S. suis* strains that were obtained

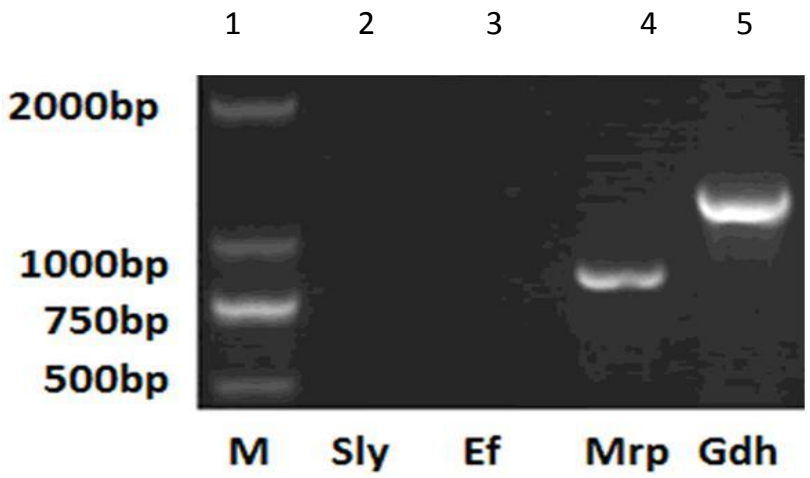

Figure 1. PCR products of the virulence-associated gene of the HW07 isolate. Lanes 2, 3, 4, 5 are PCR products using primers for *Sly, Ef, Mrp, Gdh* genes of strain HW07, respectively. Lane 1 is DL 2000 marker.

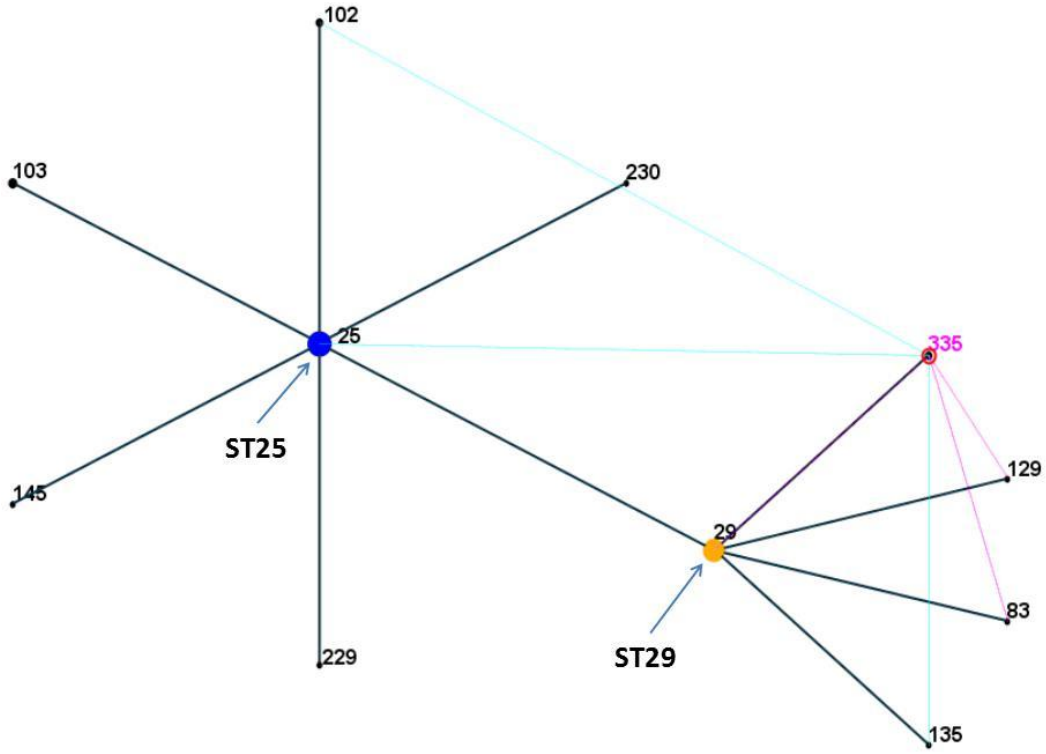

Figure 2. Analysis of the ST25 clonal complex of *S. suis*. eBURST groups were obtained from the entire *S. suis* public MLST database with the stringent (default) group definition; the eBURST group that included ST25 is displayed. The primary founder, ST25 (bootstrap confidence value of 100%), and a major subgroup founder, ST29, are labeled.

from the GenBank database. As shown in Figure 3, HW07 isolate was grouped in one branch with Chinese strain HB1001, S196 and BJ0401, but in a different branch obviously with the Chinese strain 40. The homologies for HW07 isolate and strains of HB1001, S196, BJ0401 were higher with 95.2-95.5% identity. The recA gene of HW07 isolate showed a 94.1, 93.5, 93.2, 90.4% identity with Japan strains DAT301, DAT289,

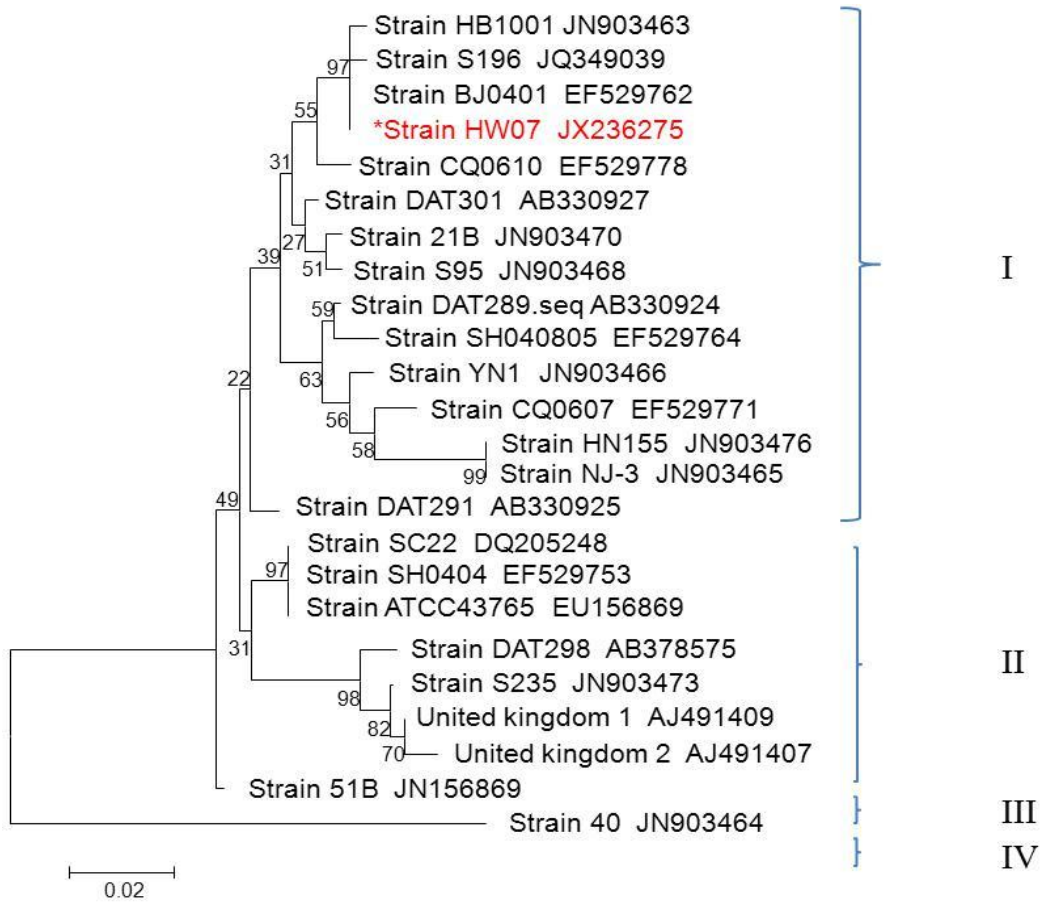

Figure 3. Phylogenetic tree between reference *S. suis* strains from the GenBank and HW07 isolate based on the nucleotide sequence of *recA* gene.

Table 3. LD_{50} of HW07 isolate and the reference strain 8074.

Strain	Infectious dose (CFU)	Total death rate	LD_{50} (CFU)
HW07	5×10^{3} - 5×10^{7}	23/50	1.25×10^{5}
8074	5×10^{3} - 5×10^{7}	10/50	3.70×10^{7}
Control		0/10	∞

DAT291 and DAT298, then 89.8 and 94.6% identity with two United Kingdom strains, respectively. There was a poor homology between the nucleotide sequence of recA gene in HW07 isolate and Canada strain ATCC43765. The lowest homology of 81.6% was found between recA genes in HW07 isolate and Chinese strain 40.

Pathogenicity test of HW07 isolate to zebrafish

In order to test the virulence of HW07 isolate, zebrafish were challenged with HW07 isolate. As shown in Table 3, Control fish injected with PBS suffered no mortality.

Clinical symptoms showed that branchia and hypogastric region of the diseased fish bleed. The SS7 were re-isolated from all organs of all dead zebrafish. Results suggest that the pathogenicity of HW07 isolate was stronger than international standard SS7 strain 8074.

Pathogenicity test of HW07 isolate to piglet

The piglets in isolate-infected showed body temperature elevated, ear purple, diarrhea and weight loss. No macroscopic lesions were observed in the control pigs. The piglets in infect showed sub-mandibular lymph node

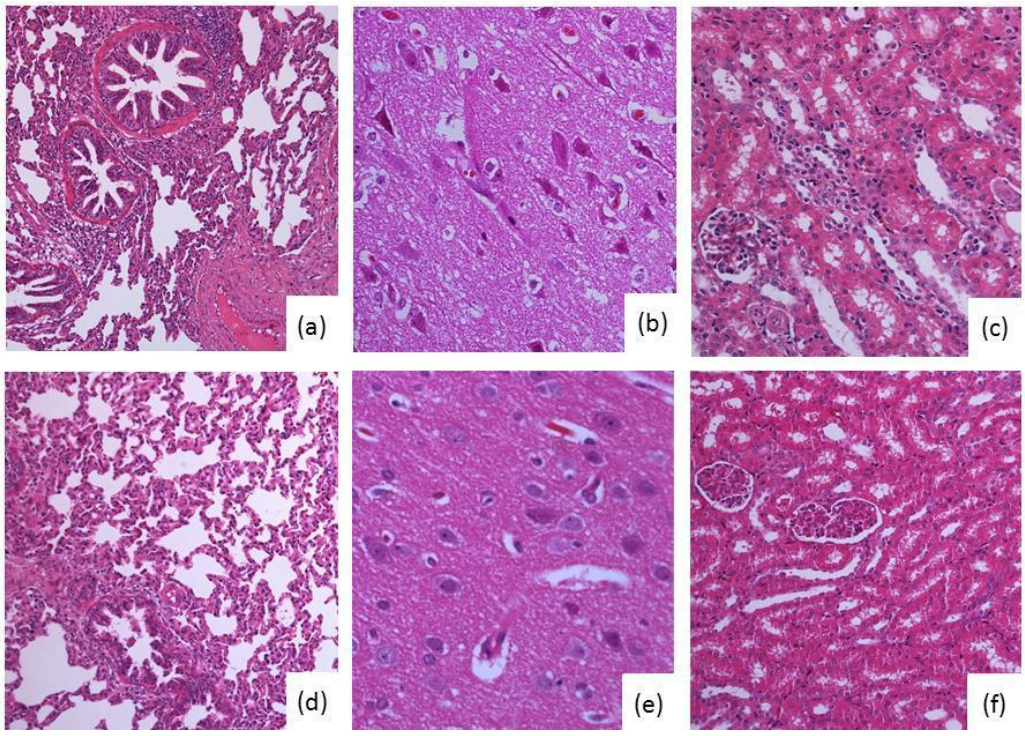

Figure 4. Pathological examination of tissues. (a) Lung-diffuse interstitial pneumonia was observed with marked thickening of alveolar septa by infiltration of lymphocytes; (b) brain edema; (c) kidney- a few of lymphocytes inflitrated in interstitial substance. (d) lung tissues from control group. (e) brain tissues from control group. (f) kidney tissues from control group. The tissues were stained with hematoxylin and eosin. Bar, 100 um.

lesions, including intumescentia and hemorrhage. The affected lung and kidney lesions appeared bleeding point on the surface. The control pigs showed no pathological lesions. The pathological brain lesions in infected pigs showed mild edema. The pathological lung lesions in infect pigs showed the following characteristics: intravascular thrombosis and widened alveolar septa with neutrophil suppurative lesions. The pathological kidney lesions showed lymphocytes infiltration (Figure 4). Bacteria that were isolated from all organs were identified as HW07 isolate.

DISCUSSION

In this research, a recent isolate come from *S. suis* infected pigs in Heilongjiang province. The identification of the isolate had been confirmed by morphological biochemical and molecular analysis. MLST was used to determine the genotype of HW07 isolate. As a result, one novel ST was found whose allelic profile was 8, 30, 5, 34, 9, 3, 25. Allelic profiles of ST129, ST83 and ST29 were the closest matches with allelic profile of HW07 isolate. From the eBURST (Figure 2), we can see that ST25 is the primary founder of this group and ST29 is the subgroup founder of this group. This group should be ST25 clonal complex in which serotype 7 strain is the main representative strain. So, ST335 belong to ST25 complex, in which strains appeared less associated with human invasive disease (King et al., 2002). The result of pathogenicity experiment hinted that the HW07 isolate was virulent. In the research of Pian et al.(2012). The *S.suis*-05ZYH33-infected piglets die after infection 2 days. Therefore, the possibility is suggested that the virulence of HW07 isolate was weaker than strain 05ZYH33 from human invasive disease, stronger than international standard *SS7* strain 8074.

The *recA* protein is composed of about 350 amino-acid residues (Karlin et al., 1995; Roca and Cox, 1997), which sequence is very well conserved among eubacterial species (Cerutti et al., 1992) and the bacteria. However, in recA housekeeping genes of HW07 isolate, 2 points of deletion at position 340 and 334 leads to 7 amino acids substitution from 112 position to 118 position (substitution Trp, Tyr, Ser, Tyr, Asn, Gly, Glu to Gly, Ile, Ile, Ter, Ter, Leu, Gly). Whether or not these 7 amino acids substitution affect virulence of HW07 isolate will be further researched in our next study.

Phylogenetic analysis for recA gene indicated that four clades of wild-type strains have existed in 23 reference *S. suis* strains. The recA gene phylogenetic relationships between HW07 isolate and other *S. suis* wild-type strains

from GenBank have been clarified in this research. In this report, HW07 isolate, identified from Heilongjiang province in 2007, displayed the highest identity with Chinese SS7 strain BJ0401 and lowest identity with Chinese strain 40 isolated from lung tissues with apparent hemorrhagic lesions in Zhejiang Province, thus, HW07 isolate can be classified into Chinese predominant genotype.

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