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Characterization of *Streptococcus suis* serotype 2 isolates from China

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ABSTRACT

Streptococcus suis (*S. suis*) is a major pathogen in the pig industry and an important zoonotic agent that causes severe invasive diseases in humans. Previous studies based on multilocus sequence typing (MLST) and the associations between sequence types and genotypes or virulence suggested that North American *S. suis* serotype 2 isolates are composed of multiple populations. This study investigated the population structure of *S. suis* serotype 2 isolates in China. We constructed a phylogenetic tree for *S. suis* serotype 2 isolates based on 16S rRNA gene typing and MLST, studied associations between clades and sources, analyzed the genotype distributions of virulence markers [muramidase-released protein (MRP), extracellular protein factor (EF), and suilysin (SLY)] in different clades, computed the selection pressures for these virulence marker genes, and verified the associations between clades and virulence. There were two primary clades (populations) in the phylogenetic tree of *S. suis* serotype 2. The two populations were associated with different tissue tropisms. The genotypic distributions and selection pressures of MRP, EF, and SLY were different between the two populations, which suggested that they had different evolutionary paths. The two populations also displayed differences in virulence in experimentally infected mice. The results provide insights into the population structure of *S. suis* serotype 2 isolates in China and suggest that *S. suis* serotype 2 clade 1 is an overlooked population that deserves further evaluation.

1. Introduction

Streptococcus suis (*S. suis*) is a major porcine pathogen and a zoonotic agent that causes severe invasive diseases in humans (Gottschalk et al., 2007). Based on the capsular type, 33 serotypes (1/2, 1–31, and 33) have been described, among which serotype 2 is most closely associated with disease. Isolates belonging to serotype 2 are predominant in most countries (Hill et al., 2005). The virulence markers most commonly used for this serotype include muramidase-released protein (MRP, encoded by *mrp*), extracellular protein factor (EF, encoded by *epf*), and suilysin (SLY, encoded by *sly*) (Fittipaldi et al., 2009; Luque et al., 2010; Wei et al., 2009).

Recently, it was reported that North American *S. suis* serotype 2 isolates display associations between their sequence types (STs) in multilocus sequence typing (MLST) and their genotypes or virulence (Fittipaldi et al., 2011). To date, the population structure of *S. suis* isolates in China is unknown. In this report, we constructed a phylogenetic tree for 180 field *S. suis* serotype 2 isolates [serotype 2

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isolates are predominant in China (Wei et al., 2009)] based on 16S rRNA gene typing and MLST. Associations between clades and sources were characterized. We sequenced and characterized the chromosome region containing *mrp*, *epf*, and *sly*; compared the genotypes of MRP, EF, and SLY in each clade, and detected the selection pressures (positive or negative) on *mrp*, *epf*, and *sly* in every clade. We also characterized the association between the clades and virulence. This study contributes to our understanding of the population structure and evolutionary paths of *S. suis* and to the development of effective measures to prevent threats to the swine industry and public health in China.

2. Materials and methods

2.1. Isolates and DNA extraction

A total of 180 *S. suis* serotype 2 field isolates were collected from different and unrelated farms in major swine production areas in 18 provinces (municipalities) in China from 2004 to 2010 (Table S1). Of the 180 isolates, 99 were obtained from the lung tissues of diseased pigs with pneumonia in which systemic infections were not observed, 80 were obtained from the tissues (brain, joints, heart, liver, or spleen) of diseased pigs with systemic infections (meningitis, arthritis, endocarditis, pericarditis, or septicemia), and 1 isolate was obtained by nasal swab from a clinically healthy pig.

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The isolates were grown in Todd–Hewitt broth medium (THB) (Oxoid, Wesel, Germany) supplemented with 10% bovine serum to mid-log phase (absorbance of 0.4 at 600 nm) at 37 °C. Genomic DNA was prepared from 4 mL of culture using the Bacteria Genomic Mini preparation kit (NewProbe, Beijing, China) as recommended by the manufacturer.

2.2. Genome sequences

The genomic sequences of the genome NCBI-registered *S. suis* serotype 2 strains (89/1591, 05HAS68, 05ZYH33, 98HAH33, SC84, p1/7, and BM407) were downloaded from the NCBI web site (www.ncbi.nlm.nih.gov/genomes). Of these strains, strain p1/7 was isolated from Britain, strain 89/1591 was isolated from Canada, strain BM407 was isolated from Vietnam, and the remaining four stains were isolated from China. The corresponding genetic sequences of the 16S rRNA gene, seven housekeeping allele genes used for MLST, and three virulence marker genes (*mrp*, *epf*, and *sly*) were used.

2.3. Construction of a phylogenetic tree based on 16S rRNA genes

PCR was used to amplify the 16S rRNA genes of the isolates using the primers described in Table S2. After the entire genetic fragment was amplified, amplicons were purified and directly sequenced using an ABI 3730 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Based on these sequences, a phylogenetic tree of the isolates and the NCBI-registered strains was constructed using the MEGA program (version 3.1) (Kumar et al., 2004) and the neighbor-joining method (Saitou and Nei, 1987).

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2.4. MLST and phylogenetic analysis

The seven housekeeping genes (*aroA*, *cpn60*, *dpr*, *gki*, *mutS*, *recA*, and *thrA*) used for MLST were PCR-amplified and sequenced as previously described (King et al., 2002). For each isolate, the alleles at each of the seven loci in the *S. suis* database (http://ssuis.mlst.net) defined the ST. Based on the concatenated MLST allele sequences, a phylogenetic tree of the isolates and the genome NCBI-registered strains was constructed as described in Section 2.3.

2.5. Characterization of the association between clades and sources

The proportion of isolate sources (pneumonia: systemic infection) among the different clades was compared using the χ^2 test. A P value of 0.05 was considered to be statistically significant.

2.6. Sequencing and analysis of mrp, epf, and sly

PCR was used to amplify the chromosome regions containing *mrp*, *epf*, and *sly*. Primers were designed from genes upstream and downstream of *mrp*, *epf* and *sly* (Table S2). After the entire genetic fragment was amplified, the amplicon was purified and directly sequenced as described in Section 2.3. PCR assay was performed as previously described (Silva et al., 2006) to determine whether *mrp*, *epf*, or *sly* was present in other loci in isolates with "mrp−", "epf−" or "sly−" type of chromosomal region sequences.

The sequences were aligned using the MEGA computer program (version 3.1) (Kumar et al., 2004) or the DNAMAN software package (version 6.0, Linnon Biosoft, Quebec, Canada). Tandem repeat sequences were determined using the Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html). The phylogenetic tree for *mrp* was constructed using the MEGA computer program (version 3.1) (Kumar et al., 2004) and the neighbor-joining method (Saitou and Nei, 1987).

2.7. Determination of selection pressures on virulence marker genes

The sequences were aligned using the Clustal X program with minor manual adjustments based on visual inspection. The number of nonsynonymous substitutions per nonsynonymous site (dN) and the number of synonymous substitutions per synonymous site (dS) were computed using the Nei–Gojobori method (Nei and Gojobori, 1986). The null hypothesis "H0: dN = dS" was tested using a Z-test in the MEGA program (version 3.1) (Kumar et al., 2004).

Different subtypes of a gene with high identity in two ends and low identity in the middle were assumed to have