

Comparative Analysis of *Streptococcus Agalactiae* S03 and S07 Isolated From *Schizothorax Prenanti* With Different Antibiotic Resistance and Virulence

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Abstract

Streptococcus agalactiae (GBS), is an important Gram-positive pathogen of fish aquaculture worldwide. In this study, we performed a comparative analysis of GBS S03 and S07 isolated from *Schizothorax prenanti* and explored the association between phenotypic antibiotic resistance as well as virulence and the genomic characteristics. Antimicrobial sensitivity tests on 12 common antibiotics using the disc diffusion method revealed that the S03 showed resistance to seven antibiotics, while S07 showed sensitivity to the tested antibiotics. Pathogenicity analysis demonstrated greater virulence of S07 than S03. Then, the occurrence of antibiotic resistance and virulence genes was identified using whole-genome sequence (WGS) of S03 and S07. There were *mefE*, *tetO*, *lnuB*, *lsaE*, *APH3'*, and *sat-4* resistance genes present only in S03 genome. And just S03 had *gyrA* and *parC* genes mutations. In addition to 51 virulence genes in both S03 and S07 genomes, S07 additionally carried virulence genes associated with invasion, such as *SAN_1519*, *rfaA* and *cylE* genes. There was complete concordance between genotypic evidence and phenotypic characteristics. Virulence factors and phylogenetic analysis showed that S03 and human sources shared an extremely close evolutionary relationship. Our findings provide important proof for using WGS as an effective tool of phenotypic predictions of GBS.

1. Introduction

Streptococcus agalactiae, or group B streptococcus (GBS), is an important Gram-positive pathogen responsible for morbidity and mortality in fish aquaculture worldwide (Evans et al. 2008; Chong et al. 2016; Kannika et al. 2017). The GBS is also an emerging pathogen associated with neonatal meningitis in humans, mastitis in cows and hematosepsis in rabbits (Bisharat et al. 2004; Ren et al. 2013; Veeraraghavan et al. 2017). To date, GBS can be classified into 10 serotypes (Ia, Ib, and II to IX), of these serotype Ia, Ib and III are regarded as the most predominant in fish GBS infection (Slotved et al. 2007; Li et al. 2013). In fish farms, transmission of GBS is possible through direct contact among individuals with cohabitation or indirectly through immersion in contaminated water of culture systems (Facimoto et al. 2017).

Whole-genome sequencing (WGS) technology has become a fast and affordable tool that is affecting research in the fields of genetics, microbiology, and ecology, as well as public health surveillance and response (Zhao et al. 2016). WGS captures the full extent of bacterial genomic diversification and allows for genome wide comparisons of clinical isolates with each other (Lee et al. 2015). Comparative genome analysis between bacterial strains which are greatly different in host specificity or virulence may help to rapidly screen for dispensable genes, gene deletions or mutations, and differentially-expressed proteins (Wang et al. 2017). Thus, WGS is an effective way of studying the mechanisms of cross-host infection, immunogenicity, pathogenicity, and resistance genotypes of bacteria. At present, hundreds of complete genomes of GBS have been sequenced. However, our understanding of the piscine GBS at the whole genome level is currently limited. Although some complete genome sequences and draft genome sequences of piscine GBS isolates have been recorded so far (Areechon et al. 2016; Facimoto et al. 2017; Liu et al. 2012; Liu et al. 2013; Pridgeon and Zhang 2014; Jaglarz et al. 2018), the correlation between

WGS analysis and phenotypic characteristics has not been reported. Here, this is the first report on draft genomes of GBS S03 and S07 isolated from *Schizothorax prenanti* with different pathogenicity and drug-resistance. Draft genomes of GBS S03 and S07 are important additions to the GenBank database of piscine GBS.

In the current study, antimicrobial susceptibility testing, experimental infection and WGS analysis were carried out to compare two GBS clinical strains isolated from *Schizothorax prenanti*. Phylogenetic analysis was performed to identify their evolutionary relationships in GBS. We attempted to assess the use of WGS to evaluate the genomic diversity and genotypic prediction of drug-resistant phenotype and pathogenicity of them.

2. Materials And Methods

2.1. Bacterial strains

GBS S03 and S07 were isolated from diseased *Schizothorax prenanti*, with characteristic clinical and pathogenic meningoencephalitis from the culture collection of the College of Veterinary Medicine, Sichuan Agricultural University, China. Serotyping assigned GBS S03 to serotype III and S07 to serotype Ia by cps cluster.

2.2. Antimicrobial susceptibility tests

Antimicrobial susceptibility to vancomycin (VAN), penicillin (PEN), cephalexin (CFX), enrofloxacin (ENR), ofloxacin (OFX), norfloxacin (NOR), tetracycline (TET), doxycycline (DOX), erythromycin (ERY), gentamicin (GEN), florfenicol (FLO) and clindamycin (CLIN) was performed by the disc diffusion method.

Antimicrobial susceptibility tests results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) breakpoints. *Escherichia coli* (ATCC 25922) was used as the reference strain for quality control according to CLSI guidelines.

2.3. Pathogenicity experiments

To confirm the pathogenicity and virulence of GBS S03 and S07, we injected the two strain suspensions into healthy *Schizothorax prenanti* and *Danio rerio*, intraperitoneally. Healthy *Schizothorax prenanti* (11.5 ± 1.2 g) and *Danio rerio* (0.4 ± 0.05 g) were purchased from Sichuan Ya-fish Company and Model Animal Research Center Of Nanjing University, respectively. 260 *Schizothorax prenanti* and *Danio rerio* were kept in 13 groups equally, respectively, in 120-L plastic tanks ($n = 20$ /tank or group) with aeration and were acclimatized at $24 \pm 1^\circ\text{C}$ for 7 days. The *Schizothorax prenanti* of 6 experiment groups were challenged with 0.1 ml of bacterial suspensions of the S03 and the other 6 groups were injected with the same volume of S07 suspensions in sterile 0.8% NaCl solution at concentrations of 1.0×10^4 , 1.0×10^5 , 1.0×10^6 , 1.0×10^7 , 1.0×10^8 , 1.0×10^9 cfu·ml⁻¹. The control fish ($n = 20$) were injected with 0.1 ml 0.8% NaCl solution. Similarly, the *Danio rerio* of test groups were injected intraperitoneally with 0.05 mL of bacterial suspensions of the S03 and S07 in sterile 0.8% NaCl solution at different concentrations mentioned

above. The control animals were injected with 0.05 mL 0.8% NaCl solution. Mortality was recorded daily for 21 d and mean lethal dose (LD₅₀) values were calculated. All the fish were euthanized by using 300 mg·L⁻¹ MS222 (yuanye Bio. Co. Ltd., Shanghai, China) for 21 d.

2.4. Whole genome sequencing and annotation

To explore further differences between S03 and S07, their draft genomes were sequenced and analyzed at whole genome level. The genomic DNA of the two strains were extracted using the TIANamp bacteria DNA kit (Tiangen, Beijing, China) and applied for library preparation using the Nextera DNA sample preparation kit (Illumina, San Diego, USA). The samples were accessed for the genome sequencing on an Illumina HiSeq™ 2000 (Illumina Inc., San Diego, USA) using a paired-end 2×100 bp protocol. The whole genome sequences were assembled using CLC Genomics Workbench 10.0 software (QIAGEN, Hilden, Germany). Contigs were initially annotated using Rapid Annotations using Subsystems Technology (RAST) and then manually checked. Resistance-related genes of the two strains were analyzed using ResFinder 2.1 server (Zankari et al. 2012). Gene clusters were classified into COG (Cluster of Orthologous Groups of proteins) categories (<http://www.ncbi.nlm.nih.gov/COG/>) according to BLAST annotations.

2.5. Identification of antibiotic resistance genes and virulence factors

To find antibiotic-resistant genes in two GBS strains genomes, known antibiotic resistance genes were downloaded from the Comprehensive Antibiotic Resistance Database (CARD) and aligned with all coding sequences (CDSs) of those genomes using BLAST (<https://card.mcmaster.ca/>). A similar method has been applied to identify virulence genes from the Virulence Factor Database (VFDB) (<http://www.mgc.ac.cn/VFs/>).

2.6. Phylogenetic tree

A phylogenetic tree of GBS strains was constructed based on seven housekeeping genes (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt*) as previously described (Jones et al. 2003). The alignments of these genes were concatenated into a single sequence alignment and a maximum likelihood tree was reconstructed using MEGA (version 10.0). The allele number and sequence type (ST) were assigned by multilocus sequence typing (MLST) website (<https://pubmlst.org/organisms/streptococcus-agalactiae>).

3. Results

3.1. General features of the GBS S03 and S07 genomes

Characteristics of the two genomes are summarized in Table 1. The circular maps of two strains showed that the genome of S03 was 2,156,979 bp in length (Fig. S1a), while that of S07 was 1,790,578 bp in length (Fig. S1b). Genome sequence of both strains showed 35% G + C contents. RAST server predicted the 2,160 and 1,641 CDSs for S03 and S07 strains, respectively. On the basis of COG classification of S03

and S07, 23.7% and 22.6% of CDSs were responsible for information storage and processing, whereas 37.5% and 39.4% were for metabolism (Fig. S2).

Table 1
General features of GBS S03 and S07.

Features	S03	S07
Host	<i>Schizothorax prenanti</i>	<i>Schizothorax prenanti</i>
Sequence type	ST19	ST891
Serotyping	III	Ia
Genome size (bp)	2,156,979	1,790,578
G + C content (%)	35	35
Total CDSs	2,160	1,641
CDSs responsible for information storage and processing (%)	23.7	22.6
CDSs responsible for metabolism (%)	37.5	39.4
Transfer RNA	42	29

The genome sequences of S03 and S07 have been deposited in the NCBI GenBank database under the accession numbers NAPX000000000 and NHZY000000000, respectively.

3.2. Antibiotic resistance profile and antibiotics resistance genes

Antimicrobial susceptibility distribution of S03 and S07 was given in Fig. 1a. The results showed that there were significant differences in resistance between the two strains. Antimicrobial tests revealed that S03 showed multidrug resistance against OFX, NOR, ENR, TET, DOX, ERY and CLIN (Table S1). By contrast, S07 was sensitive or intermediate to all 12 tested antibiotics.

The genomes of GBS S03 and S07 showed that S03 had 20 resistance genes, while S07 had 14 resistance genes (Fig. 1b and Table S2). In addition to two macrolide resistant genes (*macB* and *ermB*) and one tetracycline resistant gene *tetM*, S03 also had *mefE* and *tetO* genes. Meanwhile, S03 had additionally *InuB*, *IsaE*, *APH3'*, and *sat-4* genes not present in S07. Both strains carried genes encoding resistance to fluoroquinolone: *parC*, *gyrA*, *gyrB*, *norA* and *norB*, however, just S03 had chromosomal *gyrA* [81:S-L] and *parC* [79:S-Y] mutations (Fig. S3).

3.3. Pathogenicity experiments and virulence genes

At 21 days post-infection (dpi), GBS S07 induced higher levels of mortality and revealed a greater virulence than S03 in the pathogenicity experiments (Fig. 2). Concretely, The LD₅₀ value of S03 for *Schizothorax prenanti* and *Danio rerio* was 5.27×10⁸ cfu·ml⁻¹ and 7.09×10⁷ cfu·ml⁻¹ respectively, while that of S07 was 8.9×10³ cfu·ml⁻¹ for *Schizothorax prenanti* and 1.88×10³ cfu·ml⁻¹ for *Danio rerio*. S03 strain caused 25% and 60% mortality in *Schizothorax prenanti* at the 1.0×10⁸ and 1.0×10⁹ cfu·ml⁻¹, while S07 caused 55%, 75%, 95%, 100%, 100% and 100% at 1.0×10⁴, 1.0×10⁵, 1.0×10⁶, 1.0×10⁷, 1.0×10⁸ and 1.0×10⁹ cfu·ml⁻¹ concentrations respectively. *Danio rerio* had 20%, 35%, 55% and 70% cumulative mortality caused by S03 at 1.0×10⁶, 1.0×10⁷, 1.0×10⁸, 1.0×10⁹ cfu·ml⁻¹, while caused by S07 were 70%, 85%, 95%, 100%, 100% and 100% at 1.0×10⁴, 1.0×10⁵, 1.0×10⁶, 1.0×10⁷, 1.0×10⁸, 1.0×10⁹ cfu·ml⁻¹ respectively. There was no mortality in the control groups during the observation period.

Fifty-one virulence genes were identified in the both genomes (Table 2). Among these, *bibA*, *dltA*, *fsbA*, *pavA*, *psaA*, *srtA*, *fsbB*, *pavB*, *lap*, *lep*, *lmb* and *srr-1* were involved in attachment; *CLL_A 2400*, *hylB*, *lgt*, *mf3*, and *sugC* were involved in invasion; *cfb*, *cpsA-L*, *cpsA-Y*, *manA*, *neuA-D*, *uppS*, *rgpA*, *rmlA*, *scpA*, *rgpB*, *scpB*, *oppF*, *rgpG*, *sip*, *BC5263*, *EFD32*, *EFD32_0765* and *SMU.322c* were involved in evading/destroying host defenses; and other virulence genes, included were *coxK2*, *clpC*, *clpE*, *clpP*, *cppA*, *msbA*, *nanA*, *gbpB*, *fhuC*, *licD*, *lisR*, *lplA1*, *groEL*, *htrA/deg*, *CT396* and *stp*. Comparison of the virulence genes between S03 and S07 showed differences. S03 carried *SAG1404-1408*, *rib*, *SAK_0778–0779* and *gbs0628-0629* associated with attachment genes, while S07 were carrying *SAN_1519*, *rfbA* and *cylE* involved in invasion genes.

Table 2
Virulence genes of GBS S03 and S07.

Class	S03	S07
Attachment	<i>bibA</i> , <i>dltA</i> , <i>fsbA</i> , <i>pavA</i> , <i>psaA</i> , <i>srtA</i> , <i>fsbB</i> , <i>pavB</i> , <i>lap</i> , <i>lep</i> , <i>lmb</i> , <i>srr-1</i> , <i>rib</i> , <i>SAG1404</i> , <i>SAG1405</i> , <i>SAG1406</i> , <i>SAG1407</i> , <i>SAG1408</i> , <i>SAK_0778</i> , <i>SAK_0779</i> , <i>gbs0628</i> , <i>gbs0629</i>	<i>bibA</i> , <i>dltA</i> , <i>fsbA</i> , <i>pavA</i> , <i>psaA</i> , <i>srtA</i> , <i>fsbB</i> , <i>pavB</i> , <i>lap</i> , <i>lep</i> , <i>lmb</i> , <i>srr-1</i>
Invasion	<i>CLL_A 2400</i> , <i>hylB</i> , <i>lgt</i> , <i>mf3</i> , <i>sugC</i>	<i>CLL_A 2400</i> , <i>hylB</i> , <i>lgt</i> , <i>mf3</i> , <i>sugC</i> , <i>SAN_1519</i> , <i>rfbA</i> , <i>cylE</i>
Evading/destroying host defenses	<i>Cfb</i> , <i>cpsA-L</i> , <i>cpsA-Y</i> , <i>manA</i> , <i>neuA-D</i> , <i>uppS</i> , <i>rgpA</i> , <i>rmlA</i> , <i>scpA</i> , <i>rgpB</i> , <i>scpB</i> , <i>oppF</i> , <i>rgpG</i> , <i>sip</i> , <i>BC5263</i> , <i>EFD32</i> , <i>EFD32_0765</i> , <i>SMU.322c</i>	<i>Cfb</i> , <i>cpsA-L</i> , <i>cpsA-Y</i> , <i>manA</i> , <i>neuA-D</i> , <i>uppS</i> , <i>rgpA</i> , <i>rmlA</i> , <i>scpA</i> , <i>rgpB</i> , <i>scpB</i> , <i>oppF</i> , <i>rgpG</i> , <i>sip</i> , <i>BC5263</i> , <i>EFD32</i> , <i>EFD32_0765</i> , <i>SMU.322c</i>
Other virulence genes	<i>coxK2</i> , <i>clpC</i> , <i>clpE</i> , <i>clpP</i> , <i>cppA</i> , <i>msbA</i> , <i>nanA</i> , <i>gbpB</i> , <i>fhuC</i> , <i>licD</i> , <i>lisR</i> , <i>lplA1</i> , <i>groEL</i> , <i>htrA/deg</i> , <i>CT396</i> , <i>stp</i>	<i>coxK2</i> , <i>clpC</i> , <i>clpE</i> , <i>clpP</i> , <i>cppA</i> , <i>msbA</i> , <i>nanA</i> , <i>gbpB</i> , <i>fhuC</i> , <i>licD</i> , <i>lisR</i> , <i>lplA1</i> , <i>groEL</i> , <i>htrA/deg</i> , <i>CT396</i> , <i>stp</i>

3.4. Phylogenetic analysis

By MLST, S03 was determined to be ST19, while S07 was ST891. To investigate the phylogenetic relationships of our two isolates to other GBS strains, we used the maximum likelihood method to construct a phylogenetic tree (Fig. 3). Phylogenetic analysis was performed using 38 reported GBS strains. Interestingly, S03 was closely related to Sag158, H002 and SG-M25 strains isolated from human while S07 was grouped separately.

4. Discussion

Since 2008, GBS has been present and emerging in tilapia in China (Zhang et al. 2008). Liu et al. (2013) reported the first complete genome sequence of the piscine GBS strain isolated from cultured tilapia in China. In this study, draft genomes of GBS S03 and S07 isolated from *Schizothorax prenanti* have been recorded. The genome size of S07 was approximately 1.79 Mbp and approximately 0.37 Mbp smaller than S03. There were more predicted CDSs in the S03 genome than in S07. Genome size is the result of a balance between amplification and loss of DNA, which evolves toward an optimal state for organismal fitness (Devos et al. 2002; Lynch 2006). Furthermore, genomic changes leading to streamlined genomes with increased pathogenicity and virulence are important (Siguier et al. 2014). They might involve fragment recombination and contribute to the different virulence of S03 and S07.

Antimicrobial tests revealed that the GBS S03, showed multidrug resistance, but S07, showed drug-sensitive to the tested antibiotics. The differences in drug resistance between the two strains were significant, which may have been associated with environmental variability and/or the frequent and abusive use of chemotherapy in aquaculture (Abuseliana et al. 2010; Florindo et al. 2010). In the current study, the phenotypes of two strains were in concordance with genomic characteristics revealed by WGS. Two strains had fluoroquinolone genes: *parC*, *gyrA*, *gyrB*, *norA* and *norB*, however, just S03 had chromosomal *gyrA* [81:S-L] and *parC* [79:S-Y] mutations, explaining high-level fluoroquinolone resistance (Kawamura et al. 2003; Salma et al. 2013; Doumith et al. 2017). In addition to two macrolide resistant genes (*macB* and *ermB*) and one tetracycline resistant gene *tetM*, S03 additionally had *mefE* and *tetO*, which contributed to high-level macrolide and tetracycline resistance (Poyart et al. 2003; Doumith et al. 2017), as observed in this study. These results are consistent with what we have previously found through traditional methods (Deng et al. 2019), but WGS is clearly more efficient and accurate. Meanwhile, GBS S03 had additional *InuB*, *IsaE*, *APH3'*, and *sat-4* genes not present in S07 strain, the first two of which play an important regulatory role in lincosamide resistance (Faccone et al. 2010). The combination of *APH3'* and *sat-4*, associated with transposon Tn5405 among Gram-positive bacteria, encodes resistance to aminoglycosides (except gentamicin) and streptothricins (Derbise et al. 1997; Werner et al. 2003). These results reveal a trend towards concordance between resistance phenotype and genotypic evidence for antibiotic resistance.

The pathogenicity of the two strains were compared by intraperitoneal injection in *Schizothorax prenanti* or *Danio rerio*, which demonstrated that S07 showed much greater virulence than S03. S07 also carried more virulence genes associated with invasion, such as *SAN_1519*, *rfaA* and *cylE* genes. However, it was counter to the previous report on GBS from Tilapia in Thailand (Areechon et al. 2016), which is most likely

related to the diversity in host, area and strain. Fifty one virulence genes in both GBS S03 and S07 involved in attachment, persistence, evading host defenses, tissue penetration, and toxin-mediated diseases had been identified in the GBS genomes, providing evidence of the pathogenicity of GBS (Godoy et al. 2013; Chen et al. 2014; Kayansamruaj et al. 2014). Differences in virulence genes between the two strains are mainly reflected in the genes encoding pili, including *SAG_1404–1408*, *gbs0628-0629* and *SAN_1519*. Genes encoding pili in GBS are located within two distinct loci, denoted pilus islands 1 and 2 (PI-1 and PI-2), and comparative analysis of available genomes revealed two variants of PI-2, designated PI-2a and PI-2b (Rosini et al. 2006). *Gbs0628-0629* genes, *SAG_1404–1408* genes, and *SAN_1519* gene are located in PI-1, PI-2a and PI-2b, respectively (Rosini et al. 2006; Nobbs et al. 2008). Pili are supposed to be important virulence factors (Nobbs et al. 2008). In GBS, pilus 1 is related to the immune escape, pilus 2a confers formation of biofilms, and pilus 2b is linked to the ability of attachment and invasion (Rinaudo et al. 2010; Jiang et al. 2012). In this study, S03 carried PI-1 plus PI-2a type pilus islands while S07 carried PI-2b type pilus island, which suggested there was concordance between pathogenicity and WGS result. Previous studies showed that most of the fish-sources strains carried PI-2a type, PI-2b type or PI-1 plus PI-2b type pilus islands, while in human strains, a great number of GBS carried PI-1 plus PI-2a type or PI-1 plus PI-2b type (He et al. 2017).

Interestingly, phylogenetic analysis showed that S03 was closely related to human sources (Sag158, H002 and SG-M25) while S07 was grouped separately. Combined with genome size and phenotypic characteristics, we hypothesized that they had different origins and evolutionary directions. Further, bestowed pili types and phylogenetic analysis, we hypothesized that S03 had a common source with human GBS or the possibility of cross-infection with human strains. Unfortunately, we currently do not have adequate epidemiological information to confirm this hypothesis. Although the knowledge about the ability of GBS to cross the interspecies barrier and allow human-derived strains to infect animals, or vice versa, is poorly understood, Pereira et al. (2010) demonstrated that GBS strains from human and bovine origins could infect fish. Rajendram et al. (2016) found that human could be infected by fish-isolated GBS by consuming of raw fish. Therefore, we should attach great importance to cross-host transmission of GBS and its potential threat to public health security. In addition, whether our two strains in this study will exchange and transfer resistance genes and virulence genes in nature, with the appearance of new strains with great toxicity and extreme drug resistance, which deserve further research and attention.

5. Conclusions

In this study, GBS S03 and S07 isolated from *Schizothorax prenanti* showed greatly distinct phenotypic characteristics, including drug-resistant phenotype and pathogenicity, and they were diverse at the genome level. There was finished concordance between genotypic evidence and phenotypic characteristics. Our findings provide important proof for using WGS as an operational tool of phenotypic predictions of GBS.

Abbreviations

GBS: Streptococcus agalactiae; WGS: Whole genome sequencing; LD50: mean lethal dose; CLIN, clindamycin; ERY, erythromycin; ENR, enrofloxacin; NOR, norfloxacin; OFX, ofloxacin; TET, tetracycline; DOX, doxycycline; PEN, penicillin; CFX, cephalexin; VAN, vancomycin; GEN, gentamicin; FLO, florfenicol; dpi: days post-infection; ARDB: Antibiotic Resistance Genes Database; VFDB: Virulence Factor Database

Declarations

Ethics approval and consent to participate

All animal procedures were conducted in accordance with Animal Experiment General Requirement in China (record number GB/T 35823 - 2018) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Agricultural University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors have declared that no competing interests exist.

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Authors' contributions

Conceptualization, Lishuang Deng, Yajun Li, and Yi Geng; Data curation, Yajun Li; Formal analysis, Lishuang Deng, Yajun Li, and Yi Geng; Methodology, Lishuang Deng, Yajun Li, and Yi Geng; Supervision, Defang Chen, Yangping Ou, Xiaoli Huang, Hongrui Guo, Zhicai Zuo, Chao Huang, Zhengli Chen and Weiming Lai; Validation, Yi Geng; Writing—original draft, Lishuang Deng; Writing—review & editing, Lishuang Deng, Yi Geng, and Tayyab Rehman. All authors read and approved the final manuscript.

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Figures

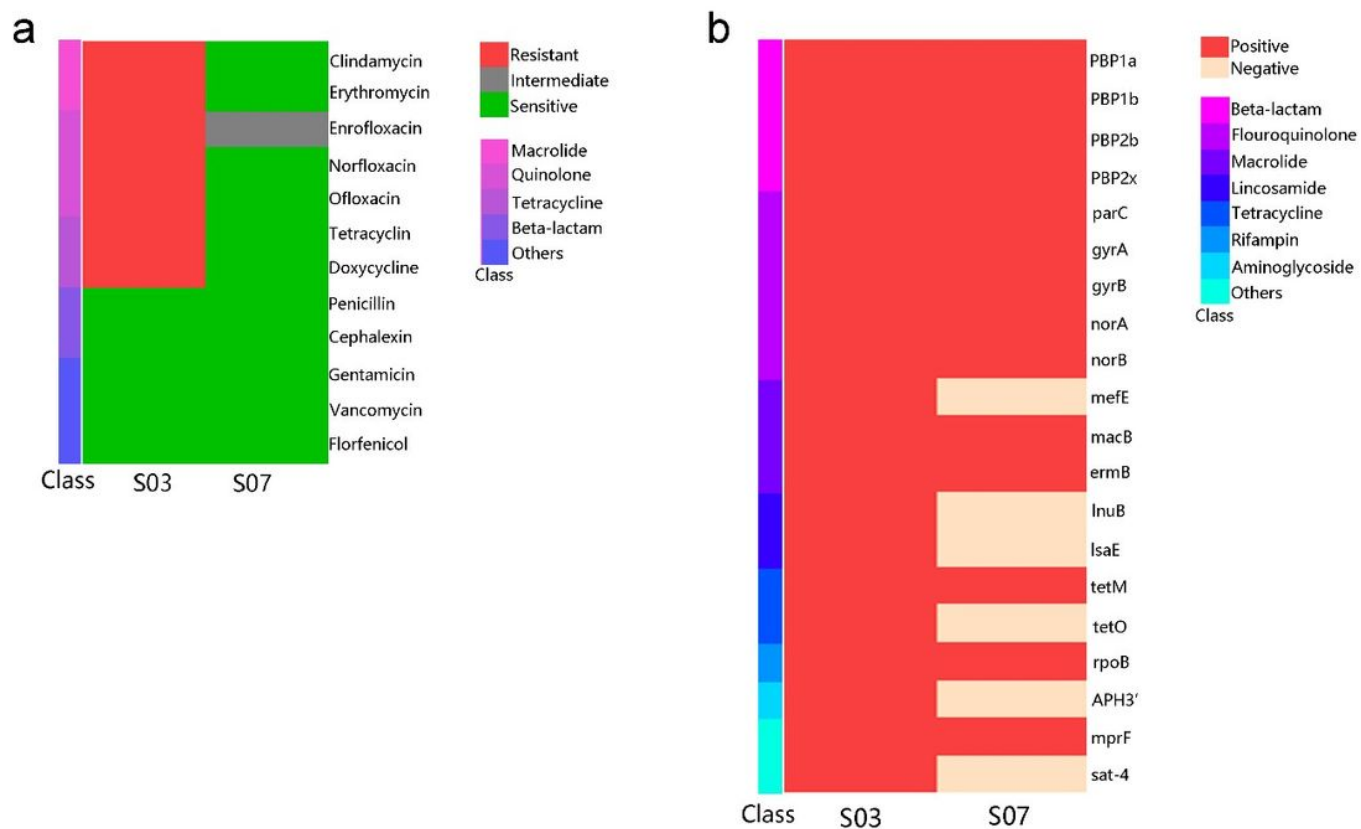


Figure 1

Heat maps of antibiotic resistance results (a) and resistance genes (b) of GBS S03 and S07, generated using graphpad (version 8.0.1).

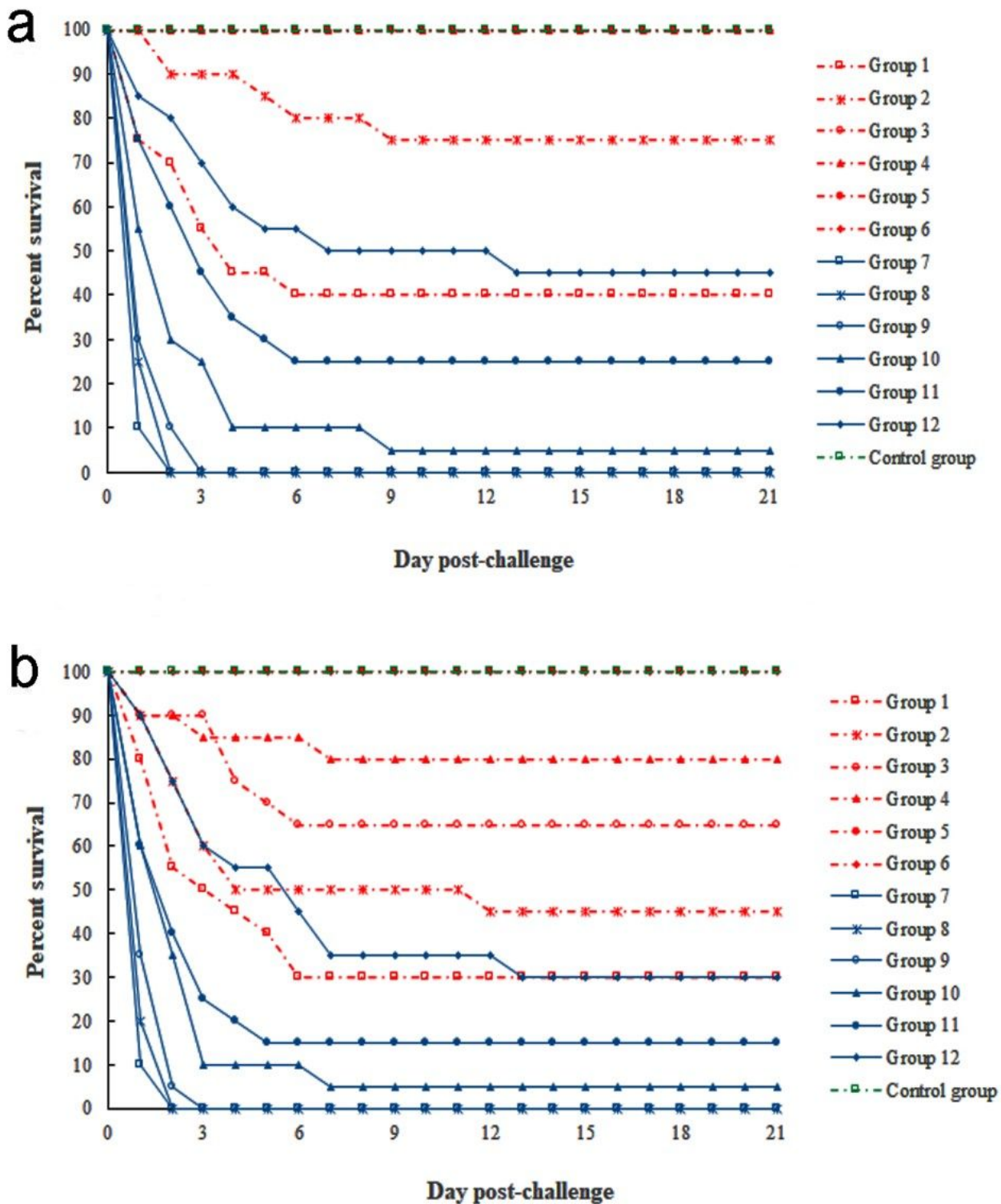


Figure 2

Percent survival (%) of *Schizothorax prenanti* (a) and *Danio rerio* (b) after challenge with different concentrations of GBS S03 and S07. Group 1-6: survival curves of fish injected with S03 suspensions at 1.0×10^9 , 1.0×10^8 , 1.0×10^7 , 1.0×10^6 , 1.0×10^5 , 1.0×10^4 cfu·ml⁻¹ concentrations respectively; Group 7-12: survival curves of fish injected with S07 suspensions at 1.0×10^9 , 1.0×10^8 , 1.0×10^7 , 1.0×10^6 , 1.0×10^5 ,

1.0×10⁴ cfu·ml⁻¹ concentrations respectively; Control group: survival curves of fish injected with 0.8% NaCl solution.

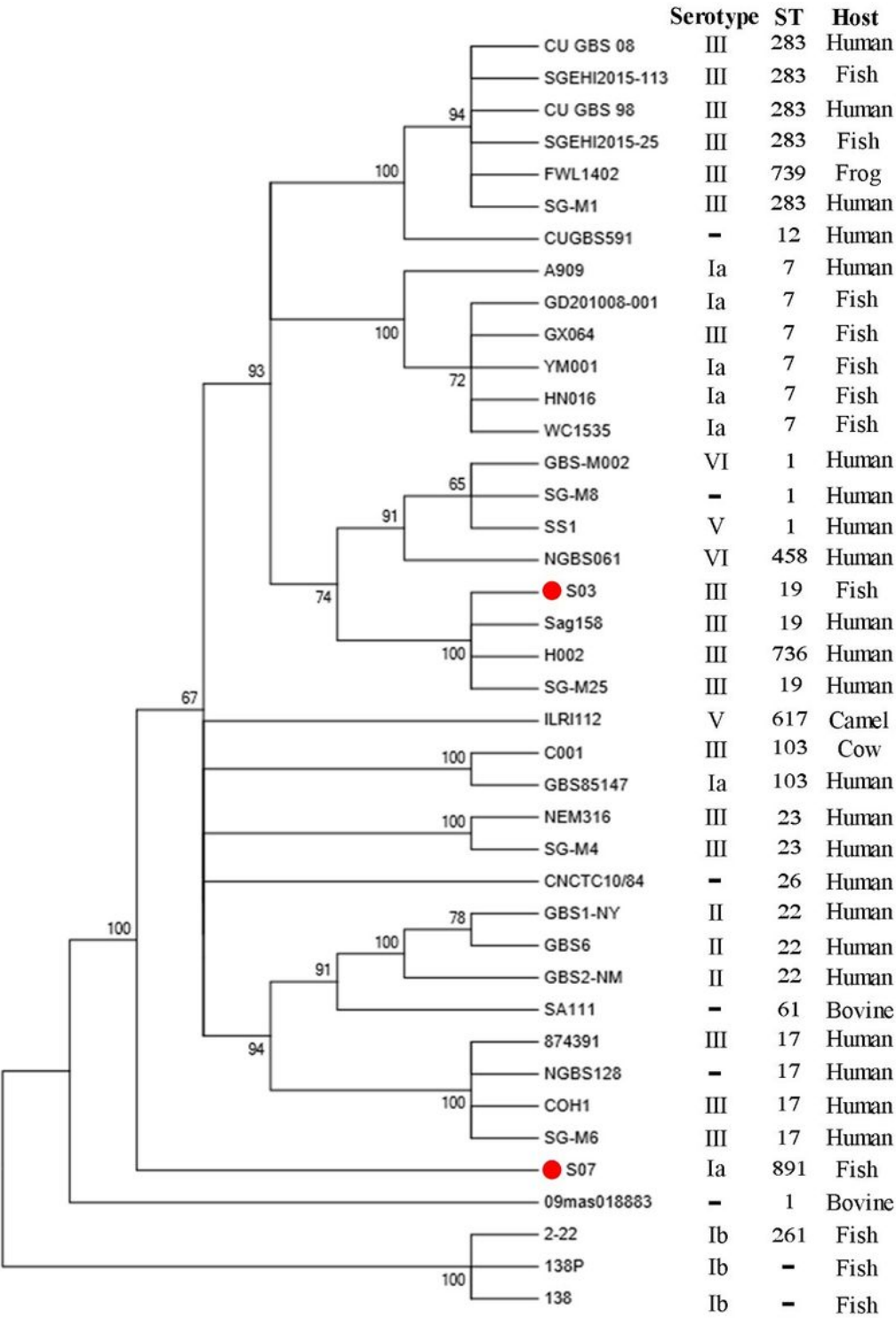


Figure 3

Molecular phylogenetic tree of the evolutionary relationship among different GBS strains using the Neighbor-Joining algorithm and 1000 bootstraps.

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