Detection of Streptococcus pyogenes virulence genes in Streptococcus dysgalactiae subsp. equisimilis from Vellore, India

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Abstract:

Streptococcus dysgalactiae subsp. *equisimilis* (SDSE), belonging to the group C and G streptococci, are human pathogens reported to cause clinical manifestations similar to infections caused by *Streptococcus pyogenes*. To scrutinize the distribution of genes coding for *S. pyogenes* virulence factors in SDSE, 255 isolates were collected from humans infected with SDSE in Vellore, a region in southern India with high incidence of SDSE infections. Initial evaluation indicated SDSE isolates comprising of 82.35% group G and 17.64% group C. A multiplex PCR system was used to detect 21 genes encoding virulence-associated factors of *S. pyogenes,* like superantigens, DNases, proteinases and other immune-modulatory toxins. As validated by DNA sequencing of the PCR products, sequences homologous to *speC*, *speG*, *speH*, *speI*, *speL*, *ssa* and *smeZ* of the family of superantigen coding genes and for DNases like *sdaD* and *sdc* were detected in the SDSE collection. Furthermore, there was high abundance (48.12% in group G and 86.6% in group C SDSE) of *scpA,* the gene coding for C5a peptidase in these isolates. Higher abundance of *S. pyogenes* virulence factor genes was observed in SDSE of Lancefield group C as compared to group G, even though the incidence rates in former were lower. This study not only substantiates detection of *S. pyogenes* virulence factor genes in whole genome sequenced SDSE, but also makes significant contribution towards the understanding of SDSE and its increasing virulence potential.

Introduction:

Streptococcus dysgalactiae subsp. *equisimilis* (SDSE) have subsumed under the name β-hemolytic group C and G streptococci for a long time and have been the closest known pathogenetic relative of *S. pyogenes* (Group A *streptococcus*) (Oster and Bisno 2000). Extensive taxonomic studies in the recent past years have differentiated most of the SDSE of human origin from the veterinary pathogens of group C and G. Considered to be non-pathogenic for many years, SDSE is now perceived as an important human pathogen (Brandt and Spellerberg 2009). There have been increasing reports about the isolation of SDSE from clinical manifestations similar to infections caused by *S. pyogenes* and in some cases SDSE has also been implicated in severe invasive infections such as necrotizing fasciitis and streptococcal toxic shock syndrome (Bradley et al. 1991; Efstratiou 1997; Humar et al. 2002; Baracco and Bisno 2006). Co-infections of SDSE with *S. pyogenes* as reported in necrotizing fasciitis may constitute a niche for the transfer of genetic material between both species (Bruun et al. 2013). In fact, SDSE strains are reported to comprise a wide variety of genes homologous to those coding for prominent virulence factors in *S. pyogenes*, including the M-protein, fibronectin-binding proteins, the C5a peptidase, as well as superantigens and DNases and (Davies et al. 2007a; Prabu and Menon 2011). These virulence factors impair the host immune system by mediating adhesion to host epithelial cells and resisting to opsonophagocytosis through the binding of fibrinogen, degradation of chemokines and chemotactic factors like IL-8, activation of non-specific T-cell sub-population, massive cytokine release, cleavage of IgG and degradation of neutrophil extra-cellar traps (NETs)(Cole et al. 2011). Several virulence factors especially superantigens, are located on mobile DNA elements, such as bacteriophages, integrated in bacterial genomes (Ferretti et al. 2001) and hence, are likely to be genetically transferred among different streptococcal species (Proft et al. 2003; Kalia and Bessen 2003).

Various studies have been conducted to identify virulence factors of *S. pyogenes* in SDSE (Tsai et al. 2014; Gherardi et al. 2014; Behera et al. 2014; Traverso et al. 2017). The present study was pursued to evaluate the virulence profiles of SDSE isolates from Vellore, a region in southern India, that has been of special attention due to high incidence rates of SDSE and *S. pyogenes* infections (Reissmann et al. 2010). Recent studies on isolates from Vellore, not only provided the epidemiological information of both streptococcal species but also showed the high invasive properties of *S. pyogenes* strains isolated from throat and skin infections compared to other geographical locations (Brahmadathan and Koshi 1989; Gladstone et al. 2003; Haggar et al. 2012; Behera et al. 2014). Co-occurrence of SDSE and *S. pyogenes* infections at higher abundances in Vellore (Brahmadathan and Koshi 1989; Haggar et al. 2012)

seems to be an ideal scenario for the inter-species exchange of genetic information. Overall, this study aims to identify genes encoding virulence associated factors of *S. pyogenes* in SDSE from Vellore and would probably be useful in studying the epidemiology of toxigenic SDSE strains circulating among group A streptococcus endemic regions.

Material and Methods:

Bacterial Cultivation:

A collection of 255 previously described *S. dysgalactiae* subsp. *equisimilis* isolates comprising of group G (n=210) and group C (n=45) streptococci was used in this study (Reissmann et al. 2010). All strains were isolated from humans infected with SDSE in the Department of Clinical Microbiology, Christian Medical College, Vellore, India. The bacterial isolates were cultivated over night at 37°C and 5% CO_2 in Todd Hewitt medium containing 0.5% yeast extract (THY).

DNA Isolation:

Genomic DNA was extracted from 15 ml overnight cultures. The cells were disrupted with zirconia beads using a FastPrep 24 (MP Biomedical, USA) at 4 m/s for 30sec. Bacterial debris was removed by centrifugation at 3000×g for 30 sec and the DNeasy Blood and Tissue Kit (Qiagen) was used as described in the supplier's instructions. The concentration of isolated DNA was determined using Nanodrop (peQLab, Biotechnologie GmBH), adjusted to 1 µg and stored at -20°C.

Multiplex PCR:

The genomes of the 255 SDSE isolates were screened for the presence of 21 *S. pyogenes* exotoxin encoding genes (*speA, speB, speC, speG, speF, speH, speI, speJ, speK, speL, speM, ssa, smeZ, sdaB, sdaD, sdc, spd3, sic, mac, scpA, spyCEP*) by a modified multiplex PCR system consisting of five independent reactions as described previously (Babbar et al. 2017). DNA of the genome sequenced *S. pyogenes* strains MGAS315 (GenBank accession no. AE014074.1), SF370 (GenBank accession no. AE004092.2), and MGAS8232 (GenBank accession no. AE009949.1) served as positive control. Genomic DNA of *Streptococcus gordonii* strain Challis GP204 (Oggioni and Pozzi 1996) was used as a negative control in all reactions. The integrity of the prepared DNA was validated by the amplification of the 16S rRNA gene using universal primers 27F and 1492R (Weisburg et al. 1991).

To confirm positive PCR reactions, two strains were randomly chosen and the amplified products were sequenced using ABI PRISM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM® 3730XL Analyzer (Applied Biosystems, USA). The sequence data was processed with BioEdit, version 7.0.1 (Isis Pharmaceuticals, Carlsbad, CA, USA) and confirmed using BLAST sequence comparison (http://blast.ncbi.nlm.nih.gov/). All generated

gene sequences were submitted to GenBank under the accession numbers MF419651-MF419684.

Results and Discussion:

There have been increasing reports on the identification of *S. pyogenes* virulence factors in *Streptococcus dysgalactiae* subsp. *equisimilis* group C and G. Among these virulence factors, genes encoding superantigens (*speA, speC, speG, speH, speI, speJ, speK, speL, speM, ssa* and *smeZ*) (Ikebe et al. 2002; Sachse et al. 2002; Igwe et al. 2003; Hashikawa et al. 2004; Davies et al. 2007b; Prabu and Menon 2011; Rato et al. 2011; Tsai et al. 2014; Gherardi et al. 2014; Behera et al. 2014; Traverso et al. 2017), C5a peptidase (*scpA*) DNases (*sdc, sdaD, sdaB*) and the IL-8 cleaving protein (*spyCEP*) (Davies et al. 2007a) have been previously reported. Keeping in view the current scenario, our study investigated the distribution of *S. pyogenes* virulence factor genes in SDSE isolated from human patients in Vellore, a region in south India with high incidence rates of these two pathogens (Reissmann et al. 2010).

Initial typing of the collected 255 SDSE isolates revealed that the majority exhibited the Lancefield group G antigen (82.35%, n=210), while the remaining 45 isolates (17.64%) belonged to the Lancefield group C. The screening clearly showed the dominance of group G SDSE strains over group C in this geographical location. Through the application of the multiplex PCR, we could detect homologs of the *S. pyogenes* genes *speC, speG, speH, speI, speL, smeZ, sdc, sdaD* and *scpA* in SDSE strains. Strains belonging to group G streptococci were seen to carry *speC* (0.9%), *speG* (1.4%), *speH* (2.8%), *speL* (2.8%), *smeZ* (5.7%), while group C SDSE isolates carried *speC* (6.6%), *speG* (80%), *speH* (2.2%), *speL* (2.2%), *smeZ* (4.4%), and in addition also *speI* (2.2%) and *ssa* (8.8%) that were exclusively detected in group C (**Figure-1; Table S1**). Even though we could detect genes encoding for DNases like *sdaD*, we were not able to track the gene for the most prevalent type of DNases in *S. pyogenes*: *speF* (also known as mitogenic factor) (Lamagni et al. 2008; Luca-Harari et al. 2009; Efstratiou and Lamagni 2016). Similarly, we could not detect the *speB* gene, a ubiquitous gene in genomes of *S. pyogenes* that encodes a broad spectrum cysteine protease (Haataja and Gerlach 2001; Nelson et al. 2011).

Comparative analysis of the identified genes with those present in 26 SDSE strains where the whole genome sequence is available (WGS) strains (Available in PATRIC database, Version 3.4.9) showed the presence of only *speG*, *sdc* and *scpA* in both strain collections (**Figure-2**). Interestingly, the relative abundance of *scpA* was comparatively high within both cohorts (55% in Vellore strains and 74% in WGS strains), however, *speG* in SDSE strains from Vellore was found to be relatively lower (15.6% in Vellore, in comparison to 55.5% in WGS strains). The

gene encoding the DNase sdc, on the other hand, was found in higher abundance in SDSE strains from Vellore (9.2%), in comparison to WGS (3.7%). Interestingly, the gene coding for streptodornase *spd3* was present in 14.8% of the WGS strains but was completely absent in the Vellore strain collection. It is also interesting to note that genes, such as *speC* (2%), *speH* (2.8%), *speI* (0.4%), *speL* (2.8%), *ssa* (1.6%), *smeZ* (5.6%) and *sdaD* (9.2%) could be detected in the isolates from Vellore but were absent in the WGS strains. However, as all those genes are in relatively low abundance in the SDSE strains from Vellore, their absence from previously sequenced SDSE strains is expected, but shows that PCR based approaches are still valuable to analyze strain collections.

To validate the detected gene homologs, the amplicons of two randomly selected strains with positive PCRs were sequenced and the percentage of homology to the corresponding genes of *S. pyogenes* was calculated. The results of sequencing are summarized in **Table-1**. All of the genes were covering a minimum of 98% of the target amplification or PCR product with 93-100% similarity in gene sequence, with exception of *speG that* was 84% similar to its corresponding gene in *S. pyogenes* but 100% similar to $speG_{dys}$, the *speG* homolog found in SDSE.

There have been reports that point towards co-infections of SDSE and *S. pyogenes*, owing to their similar clinical manifestations (Bruun et al. 2013) (Watanabe et al. 2016). It is quite possible that such co-infections provide a suitable niche for genetic transfer between both species. Calculating the relative number of exotoxin genes identified in the analyzed isolates, we found that most of the group G- SDSE isolates had either none or only one of the examined virulence genes located in their genome (**Figure-3**). Interestingly, the entire group C – SDSE isolates carried at least one of the examined virulence genes. Incorporation of virulence encoding genes of *S. pyogenes* to group C was seen to be higher than in group G. It can, therefore, be indicative of more genetic exchanges in group C and *S. pyogenes* when compared to group G.

To conclude, we detected a wide range of virulence factors including superantigens (*speC, speG, speH, speL, and smeZ*), DNases (*sdc, sdaD*) and other immune modulatory exotoxins (*scpA*) in our strain collection of Vellore, India. Additionally, we observed a greater perpetuation of *S. pyogenes*' virulence factors in group C as compared to group G SDSE. This study hence substantiates the presence of these virulence factors in SDSE from Vellore in India and helps in building better understanding of changing epidemiology and pathogenicity of SDSE.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

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274 # Tables:

275 **Table-1:** Sequencing results of PCR products of randomly selected strains of group G and
276 group C- SDSE isolates

Gene	Strain	Target Species	Strain	Best Fit [%]	Query Coverage [%]
speC	**C 147**	*S. pyogenes*	MGAS8232	100	100
	C 148	*S. pyogenes*	MGAS8232	100	100
	G 128	*S. pyogenes*	MGAS8232	100	100
	G 151	*S. pyogenes*	MGAS8232	99	100
speG$_{dys}$	**C 118**	SDSE	AC-2713	99	99
	C 119	SDSE	AC-2713	99	99
	G 93	SDSE	AC-2713	99	100
	G 96	SDSE	AC-2713	98	100
speH	**C 145**	*S. pyogenes*	SF370	100	100
	G 242	*S. pyogenes*	SF370	100	100
	G 243	*S. pyogenes*	SF370	100	100
speI	**C 153**	*S. pyogenes*	SF370	100	100
speL	**C 152**	*S. pyogenes*	MGAS8232	100	99
	C 122	*S. pyogenes*	MGAS8232	99	98
	G 135	*S. pyogenes*	MGAS8232	100	98
	G 169	*S. pyogenes*	MGAS8232	100	98
ssa	**C 122**	*S. pyogenes*	MGAS315	99	98
	C 123	*S. pyogenes*	MGAS315	98	100
smeZ	**C 134**	*S. pyogenes*	MGAS8232	87	99
	C 145	*S. pyogenes*	MGAS5005	99	100
	G 90	*S. pyogenes*	MGAS5005	98	100
	G 91	*S. pyogenes*	MGAS5005	98	100
sdaD	**C 124**	*S. pyogenes*	MGAS5005	99	100
	C 130	*S. pyogenes*	MGAS5005	98	100
	G 106	*S. pyogenes*	MGAS5005	99	100
	G 112	*S. pyogenes*	MGAS5005	100	100
sdc	**C 122**	*S. pyogenes*	MGAS315	98	99
	C 123	*S. pyogenes*	MGAS315	98	99

	G 115	*S. pyogenes*	MGAS315	100	100
	G 119	*S. pyogenes*	MGAS315	100	99
scpA	**C 118**	*S. pyogenes*	MGAS315	92	99
	C 119	*S. pyogenes*	MGAS315	99	100
	G 106	*S. pyogenes*	MGAS315	92	99
	G 112	*S. pyogenes*	MGAS315	100	99

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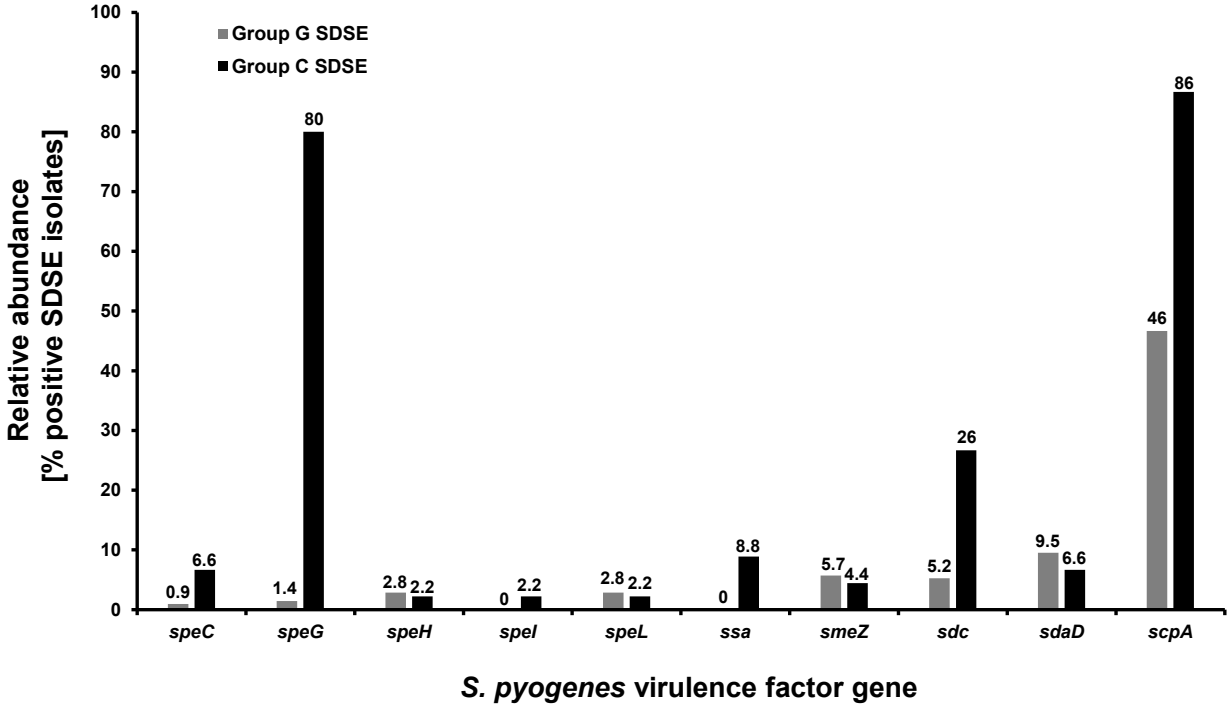
Figure Legends:

Figure-1: **Percentage abundance of all *S. pyogenes* virulence factor genes detected in group C (grey bars) and group G (black bars) SDSE isolates from Vellore.** Genes that were absent in all analyzed SDSE isolates are not depicted. Percentage abundance for all depicted genes has been mentioned above each bar.

Figure-2: **Percentage abundance of all *S. pyogenes* virulence factor genes detected in the SDSE isolates from Vellore (grey bars) in comparison to whole genome sequenced strains (Black bars) obtained from PATRIC database.** Genes that were absent in both groups are not depicted. Percentage abundance for all depicted genes has been mentioned above each bar.

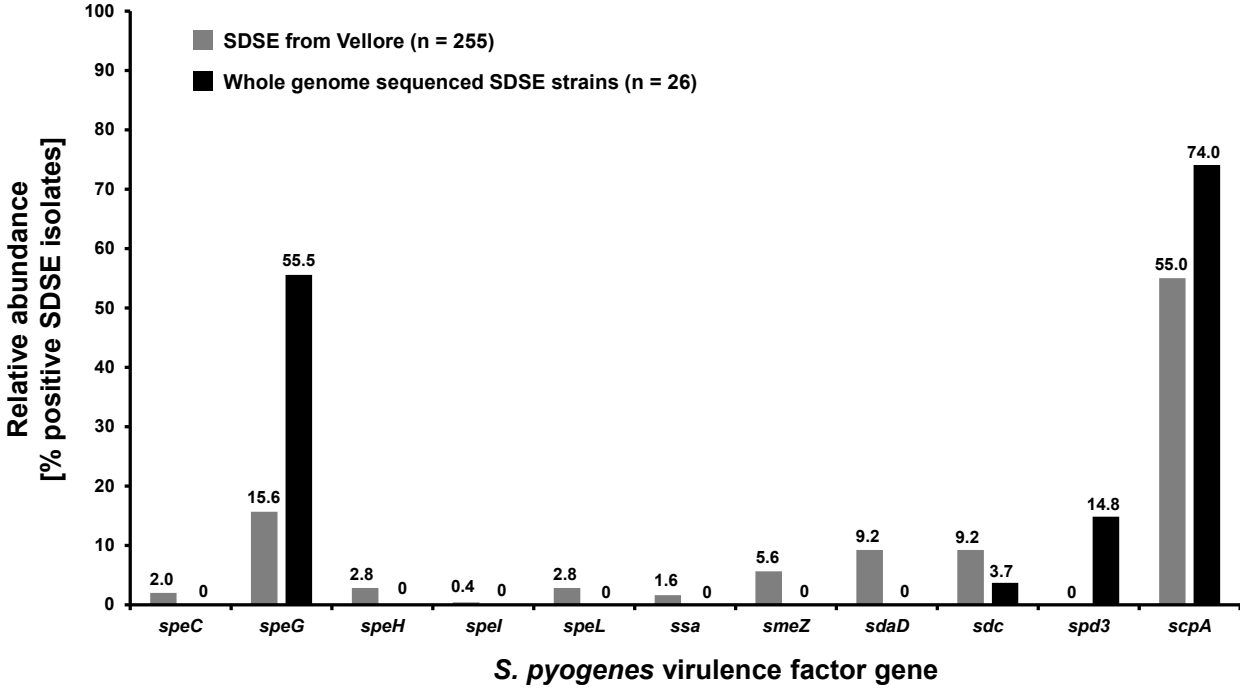
Figure-3: **Percentage abundance of group C and group G SDSE isolates from Vellore carrying different numbers of *S. pyogenes* virulence factor genes.** Total number of detected exotoxins in each isolate was calculated. A relative abundance was measured by calculating the percentage of isolate carrying a particular number of genes. (T-test, $p<0.05$)

293 **Figure-1**:



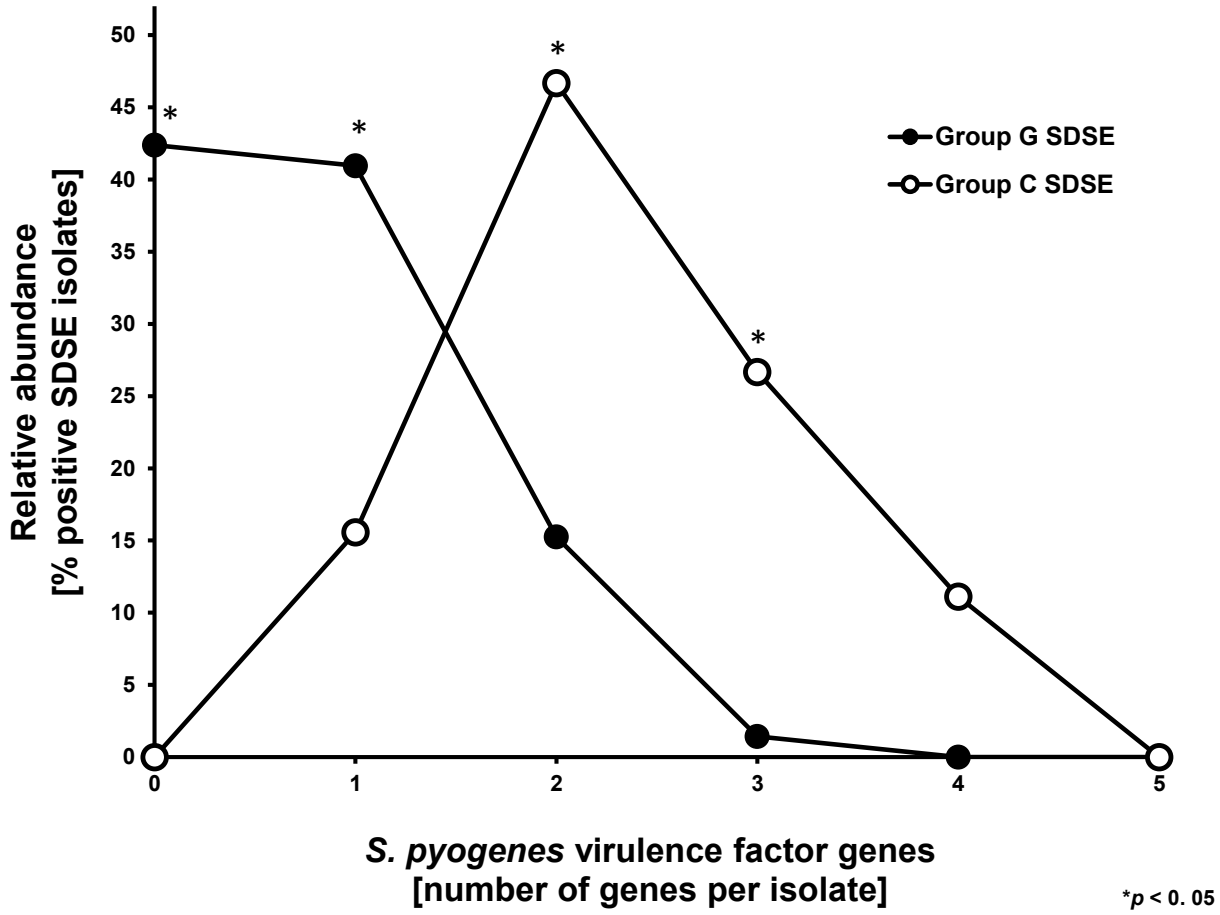
294

295 **Figure-2**:



296

297 **Figure-3**:



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