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Expression of virulence genes in Group B Streptococcus isolated from symptomatic pregnant women with term and preterm delivery

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ABSTRACT

Aims: Maternal vaginal Group B Streptococcus (GBS) colonization is considered a risk factor for preterm delivery and, consequently, neonatal infections. Previous studies have portrayed the important roles of these virulence factors, including hemolytic pigment, hyaluronidase (HylB), serine-rich protein (Srr) and bacterial surface adhesion of GBS (BsaB) in mediating GBS colonization and intrauterine ascending infection, causing preterm delivery. This study aimed to investigate the association between mRNA expression of virulence genes in GBS isolates obtained from symptomatic pregnant women and preterm delivery.
Methodology and results: GBS isolates were obtained from high vaginal swabs of 40 symptomatic pregnant women of gestational age of less than 37 weeks. RNA was extracted from these GBS isolates and RT-qPCR was performed to determine the relative mRNA expression of GBS virulence genes, including *CylE* (encode enzyme required for the biosynthesis of the hemolytic pigment), *HylB*, *Srr-1* and *BsaB*. Socio-demographic details and obstetric history were not found to be associated with the delivery outcomes of these women. The GBS isolates from symptomatic pregnant women who delivered prematurely showed a higher expression of *CylE* gene and a trend towards an elevated expression of *HylB* gene compared to women with term delivery. Meanwhile the expression of both *Srr-1* and *BsaB* genes was similar between symptomatic pregnant women who had term or preterm delivery.
Conclusion, significance and impact of study: The results suggest that following vaginal colonization, both *CylE* and *HylB* genes are likely to contribute to intrauterine ascending infection and inflammation, leading to preterm delivery in humans. These virulence factors may be targeted for the pre-clinical stages of vaccine development or therapeutic intervention.

Keywords: Group B Streptococcus, vaginal colonization, ascending intrauterine infection, preterm delivery

INTRODUCTION

The GBS are β-hemolytic Gram-positive bacteria that are found to asymptomatically colonize the gastrointestinal and/or urogenital tract of approximately 18% of pregnant women globally (Russell *et al.*, 2017; Seale *et al.*, 2017). Vaginal colonization by GBS is known as one of the risk factors of preterm delivery (Bianchi-Jassir *et al.*, 2017), as these women who are colonized are at risk for ascending intrauterine infection (Edwards *et al.*, 2019). Ascending infection is recognized as a route used by vaginal bacteria to get into the uterus through the cervix to subsequently penetrate gestational tissues, including decidua, chorion, amnion and amniotic epithelium, causing intraamniotic infection (Goldenberg *et al.*, 2000; Bastek *et al.*, 2011; Whidbey *et al.*, 2013). This is accompanied by elevation

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of proinflammatory cytokine and chemokine synthesis in the amniotic sac and early activation of mechanisms of parturition triggering preterm delivery (Bastek *et al.*, 2011; Agrawal and Hirsch, 2012). The rate of preterm delivery, which is defined as the delivery that occurs before 37 weeks of gestation, ranges from 8.1% to 11.2% in Malaysia (Jeganathan and Karalasingam, 2021). Preterm delivery affects around 11% of births globally, resulting in an estimated 15 million babies being born prematurely each year (Vogel *et al.*, 2018).

Ascending intrauterine infection may cause the transmission of GBS to the fetus in utero and the newborn during delivery via aspiration of contaminated amniotic fluid and vaginal fluids, respectively, leading to the development of early-onset neonatal infection (Verani *et al.*, 2010). Neonatal infections by GBS are classified into early-onset disease (that occurs <7 days after birth) and late-onset disease (that occurs 7-90 days postnatally). Approximately 17-20% of neonates of GBS colonized mothers are colonized and 1-7% of them will develop with invasive disease (Chan *et al.*, 2015).

Previous *in vitro* and *in vivo* studies have portrayed the important role played by certain virulence factors that include hemolytic pigment, hyaluronidase (HylB), serine-rich repeats (Srr) and bacterial surface adhesion factors of GBS (BsaB) in mediating GBS vaginal colonization and intrauterine ascending infection, leading to preterm delivery. Hemolytic pigment is a surface-associated toxin that mediates the hemolytic activity of GBS (Whidbey *et al.*, 2013). This hemolytic activity is encoded by gene products of the *cyl* operon, which encode enzymes catalyzing different steps in fatty acid biosynthesis (Armistead *et al.*, 2020; Whidbey *et al.*, 2013). The *cylE* gene encodes for N-acyl transferase, required for the biosynthesis of the pigment which contributes to the hemolytic activity of GBS (Whidbey *et al.*, 2013). The two-component regulatory (TCL) system, CovR/S is reported to negatively regulate the transcription of *cyl* genes, including *cylE* (Rajagopal *et al.*, 2006). Thus, the mutation or loss of function of CovR/S is responsible for the hyper-hemolytic phenotype observed in GBS (Rajagopal *et al.*, 2006). The hemolytic pigment has been demonstrated to induce ascending intrauterine infection, placental inflammation, and, ultimately, preterm delivery in mice (Randis *et al.*, 2014). Meanwhile, in non-human primates (NHP), hemolytic pigment mediates GBS invasion of the amniotic cavity and induction of inflammation to trigger preterm labor (Boldenow *et al.*, 2016).

The GBS hyaluronidase (HylB) is an endoglycosidase that cleaves hyaluronic acid (HA) into disaccharides (Wang *et al.*, 2014b). HA is one of the glycosaminoglycans with a high molecular weight that makes up a major component of the extracellular matrix (Wang *et al.*, 2014b). HA contributes to cell migration, cell-cell signaling and responses to injury and infection (Stern and Jedrzejas, 2006; Fallacara *et al.*, 2018). Thus, it is proposed that HylB may be required to allow the spread of the GBS from the initial site of colonization/infection (Wang *et al.*, 2014b). The GBS strains isolated from amniotic fluid of preterm labor women or blood of infected neonates expressed higher hyaluronidase activity than commensal strains obtained from rectovaginal swabs of healthy women (Vornhagen *et al.*, 2016). HylB is shown to promote ascending intrauterine infection, leading to preterm delivery in mice, possibly by dampening uterine immune response (Vornhagen *et al.*, 2016). HylB is also necessary for GBS invasion of the amniotic cavity leading to preterm labor in NHP (Coleman *et al.*, 2021).

Srr-1 and Srr-2 have a similar function but are less than 20% identical in amino acid sequence and are anchored to the GBS cell wall by sortase A (Sheen *et al.*, 2011). Srr-1 is identified in most GBS strains from serotypes Ia, Ib and V and certain strains of serotype III (Seo *et al.*, 2013). Srr-2 is only expressed by GBS serotype III, particularly in isolates belonging to multilocus sequence type 17 (ST-17), which is linked with enhanced pathogenicity and thus increased neonatal invasive diseases (Seo *et al.*, 2013). The binding regions of these proteins were discovered to attach to immobilized fibrinogen via the dock, lock and latch (DLL) mechanisms (Seo *et al.*, 2013).

FbsC is a fibrinogen binding protein anchoring to the cell wall surface via sortase A (Buscetta *et al.*, 2014). *In vitro* experiments demonstrated that it binds to immobilized fibrinogen but not to other proteins inclusive of plasminogen, fibronectin, or bovine serum albumin (Buscetta *et al.*, 2014). Sequence analysis of FbsC and the bacterial surface adhesin B (BsaB) revealed them to be identical and encoded by the same gene (Buscetta *et al.*, 2014). Another study using different strains of GBS showed that BsaB is able to bind to immobilized fibronectin and laminin (Jiang and Wessels, 2014). *In vitro* and *in vivo* studies reflect that both Srr and BsaB are adhesins important for GBS vaginal colonization (Sheen *et al.*, 2011; Jiang and Wessels, 2014; Wang *et al.*, 2014b). However, there is a limited study to demonstrate how and if these virulence genes are involved in the pathogenesis of preterm delivery in humans. The aim of this study is to investigate the association between mRNA expression of virulence genes *HylB*, *CylE*, *Bsab* and *Srr-1* in GBS isolates from symptomatic pregnant women with more than 37 weeks gestational age who undergo preterm delivery.

MATERIALS AND METHODS

Study population

A laboratory-based follow-up study on pregnant women attending the Department of Obstetrics and Gynaecology at Hospital Tengku Ampuan Afzan (HTAA) in Kuantan. Ethical approvals from IIUM Research Ethics Committee (IREC) and the Medical Research and Ethics Committee (MREC) with ethical approval numbers IREC 2021-184 and NMRR-19-1366-46490, respectively, were acquired for the use of clinical data and biological specimens from consented pregnant women for research purposes. The single proportion method formula was chosen to calculate the sample size. The most optimum sample size is 34 and

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taking into account 20% of the margin, 41 samples are required in this study.

Forty women with gestational age less than 37 weeks who presented with preterm labor, PPROM, vaginal discharge, or vaginal bleeding who had high vaginal swab samples obtained for routine cultures were recruited as study participants. Twenty-two of these GBS positive symptomatic pregnant women of had preterm delivery and 18 delivered at term. The group of women with term delivery was used as an experimental control. Written informed consent was obtained from these study participants. Symptomatic pregnant women with more than 37 weeks gestational age and/or who received antibiotics in the two weeks before the collection of high vaginal swab samples were excluded from this study.

Demographic details including maternal age, race, body mass index (BMI), education level, occupation, income, smoking habit, alcohol consumption, parity and gestational age were obtained from the symptomatic pregnant women who satisfied the inclusion/exclusion criteria. Additionally, characteristic obstetrical history, including previous preterm delivery, multiple pregnancies, habitual abortion, cervical incompetence, pathological pregnancy (gestational diabetes, pregnancy-induced hypertension, intrahepatic cholestasis of pregnancy, placenta previa, and placental abruption), and delivery outcomes were also collected.

Clinical definitions

Preterm labor is defined as the onset of labor characterized by regular uterine contractions (3 to 4 contractions lasting 30-45 sec in 10 min) accompanying cervical changes (cervical dilatation and cervical effacement) that occur before 37 weeks of gestation (Lin *et al.*, 2001). Preterm contraction is defined as uterine contractions that occur before 37 weeks of gestational age (Lin *et al.*, 2001). PPROM is defined as the spontaneous rupturing of fetal membranes before 37 weeks of gestation and before the onset of labor.

Processing of GBS isolates

High vaginal swab samples obtained from these symptomatic pregnant women were sent to the Department of Pathology of HTAA for GBS isolation and identification. The swab samples were inoculated on blood agar, incubated aerobically for 24 h and GBS colonies that exhibited beta hemolysis (a small zone of hemolysis around each colony) were differentiated from other beta-hemolytic organisms. CAMP test was used for further identification of GBS. The GBS-positive isolates were then collected and transported immediately in an insulated transport box to the Research Microbiology Laboratory, Basic Medical Sciences, Kulliyyah of Medicine, IIUM, for further processing.

Reverse transcription- quantitative PCR (RT-qPCR) to determine the expression of GBS virulence genes

The GBS isolates were grown to log phase in Todd Hewitt Broth for 2 h before RNA was extracted from these GBS isolates using Reliaprep™ RNA Cell MiniPrep System (Promega, Wisconsin, USA) method. The extraction was carried out using the manufacturer's protocols for Gram-Positive bacteria extraction. RNA purity and concentration were determined by the A_{260} and A_{280} measurements using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and then stored at –80 °C. The RNA integrity was verified by agarose gel electrophoresis to ensure that the 23S and16S bands were visible on the gel image captured using Gel Doc-EZ imager (Bio-Rad Laboratories).

RNA was reverse transcribed using GoScript Reverse Transcriptase Master Mix (Promega, Wisconsin, USA) in a volume of 20 µL using the protocols provided by the manufacturer. The complementary DNA (cDNA) obtained was then used for subsequent qPCR. Primer sequences for the virulence genes (Table 1) were optimized and tested for their amplification efficiency in the house.

Each RT-qPCR reaction contained 2 µL of cDNA template (8 ng/µL) and 18 µL of a master mix consisting of GoTaq® qPCR Master Mix (Promega, Wisconsin, USA), forward and reverse primers, and nuclease-free water. Non-template control samples containing water substituted in place of cDNA were included in all assays to confirm the absence of a non-specific amplification product. The RT-qPCR reaction was performed in a qPCR CFX96 Real-Time system (Bio-Rad, Berkeley, USA) under the following conditions: 2 min at 95 °C, 15 sec at 95 °C, 1 min at 60 °C for *HylB* and *RecA* genes, 1 min at 55 °C for *CylE*, *Srr-1* and *Bsab* genes and finally, 1 min at 60 °C. The reaction was repeated for 40 cycles, with a melt curve at 65 °C to 95 °C with 5 sec per step. Melt curve analysis was included to ensure the lack of amplification of non-specific products for all primer sets. All the data were analyzed using Bio-Rad CFX Manager software (Version 3.1).

Once the Ct value for each sample was determined, the relative expression of *HylB*, *CylE*, *Bsab* and *Srr-1* genes against the housekeeping (reference) gene, *RecA* (Florindo *et al.*, 2012) was calculated using the $\Delta\Delta$Ct method (Livak and Schmittgen, 2001): mRNA level = $\text{Log}_2 - (\text{Ct}_{Bactin} - \text{Ct}_{Target\ gene})$

Statistical analysis

All data were analyzed using the IBM SPSS Software Version 26, for Windows 10. Chi-square test was used to determine associations of categorical variables. In case where the cell count is small (n<5), the Fischer's exact test was substituted. Continuous variables were tested for normality using the Shapiro Wilk test. One-way ANOVA was applied in normally distributed variables and Mann Whitney test or Kruskal Wallis test to not normally distributed variables. The data were presented as mean ± SEM. A $p<0.05$ was considered statistically significant.

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Table 1: Sequences, product length, concentrations and GeneBank accession numbers for RT-qPCR primers.

Gene	Primer sequences	Product length (bp)	Primer concentrations (μM)	Gene bank accession number
RecA	Forward 5'-3' AAGTTGCTCCACCATTCCGT Reverse 3'-5' TCACCCGTGCGAGAAATACC	70	0.25 μM	AF307982
HylB (Otaguiri *et al.*, 2013)	Forward 5'-3' TGTCTCCGAGGTGACACTTGAACT Reverse 3'-5' TGTGTTGTGACGGGTTGTGGATG	124	0.25 μM	U15050
CylE (Kayansamruaj *et al.*, 2014)	Forward 5'-3' TTCTCCTCCTGGCAAAGCCAGC Reverse 3'-5' CGCCTCCTCCGATGATGCTTG	124	0.25 μM	AF093787
Bsab (Jiang and Wessels, 2014)	Forward 5'-3' ACCTGTGAACGCTAAAGCTG Reverse 3'-5' GCTGACCACTTGTCACCTCT	143	0.25 μM	AL766847
Srr-1 (Liu *et al.*, 2014)	Forward 5'-3' CTCGTTCTTCTGTCTATCGTCTG Reverse 3'-5' ATGCGATATTCGTCACCTACAA	103	0.25 μM	CP063198

RESULTS

Demographics and obstetrical characteristics of the study population

Among the 40 GBS-positive symptomatic pregnant women recruited for the study, 22 (55%) delivered prematurely, while 18 (45%) had normal term delivery. The demographic details such as maternal age, race, BMI, education level, occupation, income, smoking habit, parity and gestational age were not associated with preterm delivery (all $p>0.05$, Table 2). Meanwhile, obstetric history such as previous preterm delivery, multiple pregnancies, gestational diabetes and placenta previa did not exhibit a significant association with preterm delivery ($p>0.05$, Table 2). The above results may reflect those demographic and obstetrical characteristics are probably not the major confounding factors for preterm delivery in these GBS positive symptomatic pregnant women.

Association between expression of GBS virulence genes in GBS isolates from symptomatic pregnant women with preterm delivery

In order to determine the association between the expression of the GBS virulence genes with preterm delivery, the RNA was extracted from all the GBS isolates obtained from high vaginal swabs of symptomatic pregnant women. Subsequently, the relative expression of *HylB*, *CylE*, *Bsab* and *Srr-1* genes was analyzed using RT-qPCR. The pregnant women were followed up to and soon after their estimated delivery date to inquire about the delivery outcomes. It was found that 18 (45%) and 22 (55%) of these symptomatic pregnant women had term and preterm delivery, respectively.

A trend towards significant elevation in *HylB* gene expression ($p=0.056$, Figure 1A) was seen in GBS isolated from symptomatic pregnant women who delivered prematurely as compared to GBS from women who delivered at term. GBS from symptomatic pregnant women with preterm delivery portrayed a 55% increase in expression of *CylE* gene ($p=0.004$, Figure 1B as opposed to those from women with term delivery. GBS from symptomatic pregnant women who had preterm delivery presented the same levels of *BsaB* ($p=0.643$, Figure 1C) and *Srr-1* ($p=0.719$, Figure 1D) expressions as those from women who had term delivery. Thus, these results show that among symptomatic pregnant women, there is a significant association between the expression of virulence gene *CylE* with preterm delivery. Meanwhile, there is a noticeable trend towards, albeit short of significant association observed between *HylB* gene expression with preterm delivery.

Preterm labor and PPROM are known to be precursors for preterm delivery. In our study, the majority of recruited pregnant women presented with preterm labor (n=26). Notably, among those with preterm labor, 26.9.5% (n=7) presented together with PPROM, 3.8% (n=1) with vaginal discharge and 19.2% (n=5) with vaginal bleeding. In total, there were 35% (n=14) of the pregnant women presented with PPROM. In order to investigate the association between the GBS virulence gene expression between those who had preterm delivery and term delivery in preterm labor and PPROM patients, the subjects were categorized into two groups; Group 1: those with preterm labor and PPROM who had preterm delivery. Group 2: those with preterm labor and PPROM

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Table 2: Demographics and clinical characteristics of GBS-positive pregnant women <37 weeks gestation with term and preterm delivery.

Demographics and clinical characteristics	GBS-positive pregnant women with		*p*
	term delivery (n=18)	preterm delivery (n=22)	
Maternal age (years)			0.983
≤19 (n=2)	1	1	
20-24 (n=6)	2	4	
25-29 (n=14)	6	8	
30-34 (n=12)	6	6	
≥35 (n=6)	3	3	
Race			1.000
Malay (n=37)	17	20	
Chinese (n=3)	1	2	
Indian (n=0)	0	0	
BMI			0.538
<18.5 (underweight) (n=2)	0	2	
18.5-24.9 (normal) (n=12)	6	6	
25-29.9 (overweight) (n=14)	5	9	
≥30 (obese) (n=12)	7	5	
Education levels			1.000
No formal education (n=1)	0	1	
Primary (n=0)	0	0	
Secondary (n=14)	6	8	
Tertiary (n=25)	12	13	
Occupation			1.000
Housewife (n=20)	9	11	
Employed (n=20)	9	11	
Income (RM)			1.000
B40 (<RM4,360) (n=34)	15	19	
M40 (>RM4,360-RM9,619) & T20 (>RM9,619) (n=6)	3	3	
Smoking habit			0.624
Smoker (n=1)	1	0	
Passive smoker (n=18)	7	11	
Non-smoker (n=21)	10	11	
Parity			0.253
0 (nulliparous) (n=11)	3	8	
1 (primiparous) (n=10)	6	4	
2-4 (multiparous) (n=17)	9	8	
≥5 (grand-multiparous) (n=2)	0	2	
Gestational age (weeks)			0.282
22-24 (n=1)	0	1	
25-27 (n=3)	3	0	
28-30 (n=3)	1	2	
31-33 (n=12)	4	8	
34-36 (n=21)	10	11	
Previous preterm delivery			0.105
Yes (n=7)	1	6	
No (n=33)	17	16	
Previous multiple pregnancy			1.000
Yes (n=2)	1	1	
No (n=38)	17	21	
Gestational diabetes			1.000
Yes (n=11)	5	6	
No (n=29)	13	16	
Placenta previa			1.000
Yes (n=1)	0	1	
No (n=39)	18	21	

Note: Chi-squared test was applied, and Fischer's exact test was applied in cell <5. The level of significance was set at 0.05.
Abbreviations: BMI, Body Mass Index; RM, Ringgit Malaysia.

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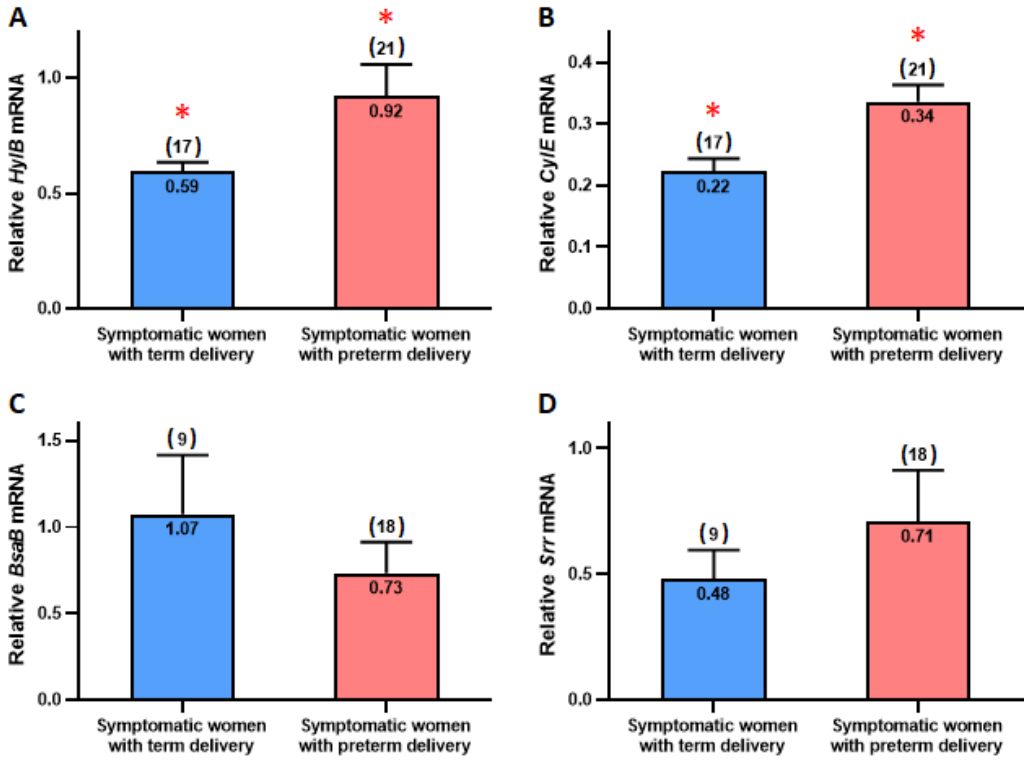

Figure 1: RNA was extracted from GBS isolates obtained from high vaginal swabs of symptomatic pregnant women and the delivery outcomes of these women were determined. Relative expression of the *HylB* (A), *CylE* (B), *BsaB* (C) and *Srr-1* (D) virulence genes were quantified using RT-qPCR normalized to *RecA* gene. Data are shown as mean ± SEM. The normality of the relative gene expression was determined using the Shapiro Wilk test and the Mann-Whitney U test was applied when the data was not normally distributed. *indicates statistical significance ($p \leq 0.05$) between symptomatic pregnant women with term and preterm delivery.

who had term delivery.

The GBS isolates from Group 1 were depicted with a trend towards a higher expression of *HylB* (p=0.062, Figure 2A) in comparison to those isolates from Group 2. A 46% increase in *CylE* expression (p=0.004, Figure 2B) was found in isolates from Group 1 as compared to Group 2. *BsaB* (p=0.854, Figure 2C) and *Srr-1* (p=0.903, Figure 2D) mRNA levels were not significantly different between the two groups. Thus, for women with preterm labor and PPROM, an association is portrayed between the expression of *CylE* with preterm delivery (Group 1).

DISCUSSION

The American College of Obstetricians and Gynaecologists (ACOG), Centres for Disease Control and Prevention (CDC) and the American Academy of Paediatrics (AAP) have established a universal culture-based screening guideline for pregnant women at 35-37 weeks of pregnancy (Schrag and Verani, 2013). Women who are tested positive will be given intrapartum antibiotic prophylaxis (IAP) (Schrag and Verani, 2013). This strategy of limiting the use of IAP to culture-positive pregnant women is an effort to reduce the outcome burden of GBS infection on pregnant mothers as well as neonates (Schrag and Verani, 2013). The implementation of this IAP program in the United States reduced the burden of early-onset GBS infections from 0.37 per 1000 live births in 2006 to 0.23 per 1000 live births in 2015 (Nanduri *et al.*, 2019). However, the IAP program had no effect on the late-onset neonatal GBS diseases, which continue as the leading cause of neonatal morbidity and mortality (Hahn *et al.*, 2021). Furthermore, the widespread use of IAP has raised concern over the perturbed gut microbiome and the emergence of antibiotic resistance in infants (Nogacka *et al.*, 2017; Tapiainen *et al.*, 2019). The development of a GBS vaccine administered to pregnant women in inducing the transfer of protective antibodies to their infants via the placenta is seen to be crucial, as it can potentially decrease the rate of preterm delivery and neonatal GBS infections, especially in low-and middle-income countries where IAP program is not feasible (Vekemans *et al.*, 2019; Berner, 2021). This approach can also be cost-effective when implemented in addition to the IAP program to reduce the GBS burden (Hahn *et al.*, 2021). However, the role of other virulence factors, especially those that are important for the mechanisms underlying vaginal colonization and ascending intrauterine infection, should be studied to further propagate vaccine development. In addition, these

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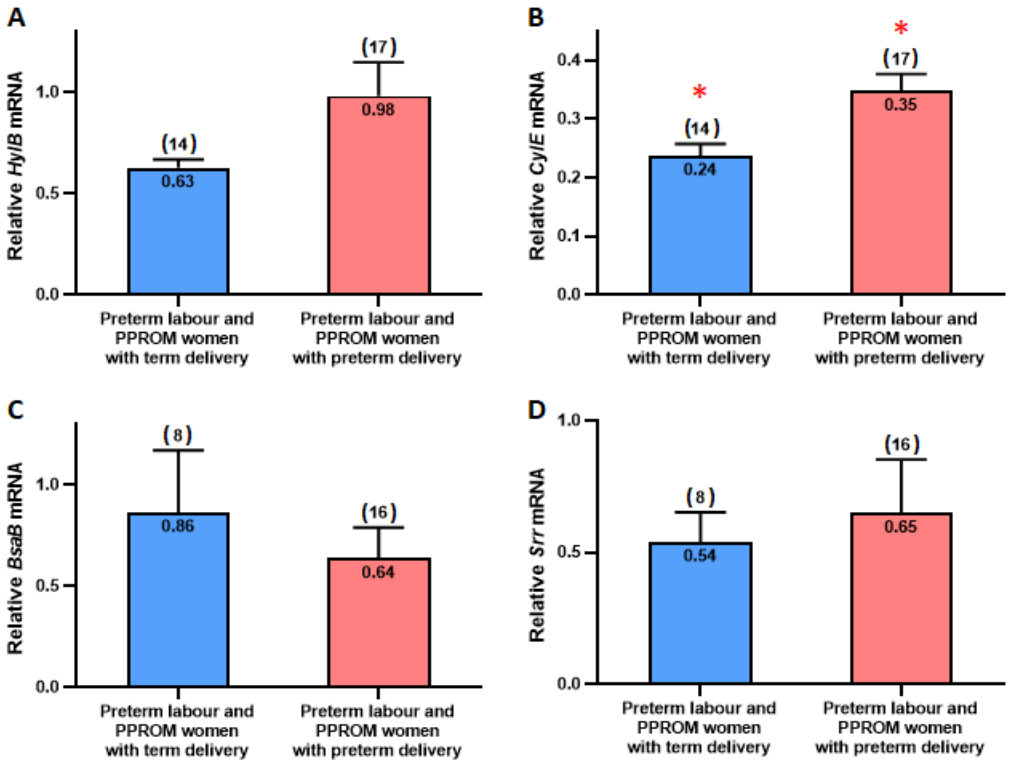

Figure 2: RNA was extracted from the GBS isolates from preterm labor and PPROM women, and the delivery outcomes were determined. The relative expression of *HylB* (A), *CylE* (B), *BsaB* (C) and *Srr-1* (D) genes were subsequently quantified and normalized against the *RecA* gene. Data are shown as mean ± SEM. The relative gene expression was tested for normality using the Shapiro Wilk test and the Mann-Whitney U test was applied when the data was not normally distributed. *indicates statistical significance (p<0.05) between preterm labor women and PPROM with term and preterm delivery.

virulence factors might serve as potential therapeutic targets.

In this study, we investigated the association between mRNA expression of virulence genes *HylB*, *CylE*, *Bsab* and *Srr-1* in GBS isolates from 40 colonized symptomatic pregnant women with <37 weeks gestation and preterm delivery. The delivery outcomes of these GBS-colonized symptomatic pregnant women were found not to be influenced by probable confounding factors related to demographic findings or obstetric history. GBS isolates from symptomatic pregnant women who delivered prematurely showed a higher expression of the *CylE* gene and a trend towards significantly increased expression of the *HylB* gene as compared to women with term delivery. GBS isolates from preterm labor and PPROM women with preterm delivery were portrayed with elevated expression of *CylE* gene and a trend towards a significantly higher expression of the *HylB* gene in comparison to women with term delivery.

Our results are supported by a previous experimental animal study showing a reduced incidence of preterm delivery and intrauterine fetal demise when the day-13 pregnant mice were inoculated intravaginally with *CylE* deficient strain as compared to wild-type GBS (Randis *et al.*, 2014). In this mice model, the hemolytic pigment was also reported to promote placental inflammation as well as maternal and fetal GBS bacteremia (Randis *et al.*, 2014). However, mice showed dissimilarities to many aspects of a human pregnancy and thus, NHP is used for studies related to human pregnancy as the closest animal model. Interestingly, inoculation of hyper-hemolytic GBS in the choriodecidual space of the pregnant NHP, was shown to cause microbial invasion of the amniotic fluid cavity (MIAC), preterm labor and fetal sepsis as compared to NHP given with GBS *covR* and *cylE* mutants (nonhemolytic GBS) or saline (Boldenow *et al.*, 2016). NHP administered with hyper-hemolytic GBS was also demonstrated with a higher level of inflammatory cytokines in amniotic fluid, recruitment of neutrophils into chorioamniotic membranes, accompanied by the formation of neutrophil extracellular traps (NETs) (Boldenow *et al.*, 2016). Similarly, *in vitro* experiments portrayed that the invasion of human amniotic epithelial cells (hAECs) and synthesis of proinflammatory cytokines from these cells were diminished when incubated with GBS strain lacking of *cylE* as compared to isogenic hyper-hemolytic or wild-type (Whidbey *et al.*, 2013). *In vitro* and *in vivo* studies revealed that hemolytic pigment mediates GBS resistance to killing by macrophages and

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neutrophils, by causing cytotoxicity of these phagocytes (Liu *et al.*, 2004; Boldenow *et al.*, 2016).

When mice were inoculated intravaginally with GBS, HylB was shown to be required for ascending GBS infection, preterm delivery and fetal demise, possibly by dampening uterine immune responses (Vornhagen *et al.*, 2016). To support this, vaginal inoculation of mice with *hylB* deficient GBS was shown to induce the production of proinflammatory cytokines in the uterus when compared to wild-type GBS (Vornhagen *et al.*, 2016). It has been shown from *in vitro* and *in vivo* experiments that HylB promotes the degradation of HA into disaccharides that can block the activation of TLR2/4 signaling and thus inflammation (Kolar *et al.*, 2015). Meanwhile, both host-derived HA fragments and other TLR2/4 ligands were demonstrated to activate TLR2/4 and thus induce the secretion of proinflammatory cytokines (Kolar *et al.*, 2015).

In the latest study by Coleman *et al.* (2021), *hylB* deficient GBS was demonstrated to cause a lower rate of microbial invasion of the amniotic fluid cavity (MIAC), preterm labor and fetal bacteremia when inoculated in the choriodecidual space of the pregnant NHP, as compared to isogenic *hylB* proficient GBS or saline (Boldenow *et al.*, 2016). This is accompanied by the diminished recruitment of neutrophils into chorioamniotic membranes and the synthesis of matrix metalloproteinase (MMPs) and prostaglandins in amniotic fluid and uterus. HylB also contributes to the GBS resistance to killing neutrophils by diminishing the production of reactive oxygen species from these immune cells as a result of HylB mediated dampening of TLR2/4 signaling, as explained above. Thus, the results from our study and previous studies reflect that both hemolytic pigment and HylB are important for GBS ascending intrauterine infection that eventually triggers preterm delivery. However, in our study, we do not have information about the GBS invasion of amniotic fluid of these symptomatic pregnant women who were vaginally colonized with GBS. It is because MIAC is not a routine procedure to determine the presence of infection, especially among women with preterm labor and PPROM in Malaysia.

Previously, it was demonstrated that both Srr-1 and Srr-2 were crucial for bacterial adherence to human vaginal (VK2/E6E7), ectocervical (Ect1/E6E7) and endocervical (End1/E6E7) epithelial cell lines (Sheen *et al.*, 2011; Wang *et al.*, 2014a). This GBS adhesion to these cells *in vitro* was enhanced by binding of Srr proteins to fibrinogen, possibly via DLL mechanisms (Wang *et al.*, 2014a). Meanwhile, *in vivo* mouse model of GBS vaginal colonization revealed that Srr-1 and its latching domain were necessary for GBS persistence in the vagina (Sheen *et al.*, 2011; Wang *et al.*, 2014a). FbsC/BsaB also contributed to GBS adhesion of human vaginal epithelial cells (VK2) and biofilm formation (Jiang and Wessels, 2014). Overall, these results signify the involvement of this virulence factor in vaginal colonization. However, in our study, the expression of both *Srr-1* and *BsaB* genes were similar between symptomatic pregnant women with term and preterm delivery. Meanwhile, GBS isolates from preterm labor and PPROM women with preterm delivery had similar expression of both *Srr-1* and *BsaB* genes to preterm labor and PPROM women who delivered at term. One possible explanation is the expression of other GBS adhesins, including pili, plasminogen-binding surface protein (PbsP), fibronectin-binding protein (SfbA) and BibA, suggested to be important for vaginal colonization (Santi *et al.*, 2009; Sheen *et al.*, 2011; Mu *et al.*, 2014; Cook *et al.*, 2018) might be upregulated in women who delivered prematurely.

The public hospitals in Malaysia do not routinely screen pregnant women for GBS infection. Only pregnant women who present with symptoms and significant previous obstetric history would warrant high vaginal swabs to be taken. Since, our study was conducted in HTAA as a tertiary hospital, we had to exclude asymptomatic pregnant women. It would be ideal to compare the expression of these virulence genes in GBS isolates obtained from asymptomatic and symptomatic pregnant women in association with preterm delivery.

CONCLUSION

In conclusion, the obtained results may suggest that following vaginal colonization, the expression of both *cylE* and *HylB* genes are upregulated to likely mediate ascending intrauterine infection and inflammation, leading to preterm delivery in humans. These virulence factors may be targeted for the exploratory and pre-clinical stages of vaccine development or therapeutic intervention to prevent invasive GBS diseases to both pregnant mothers and their newborns.

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REFERENCES

Agrawal, V. and Hirsch, E. (2012). Intrauterine infection and preterm labor. *Seminars in Fetal and Neonatal Medicine* **17(1), 12-19.**

Armistead, B., Whidbey, C., Iyer, L. M., Herrero-Foncubierta, P., Quach, P., Haidour, A., Aravind, L., Cuerva, J. M., Jaspan, H. B. and Rajagopal, L. (2020). The *cyl* genes reveal the biosynthetic and evolutionary origins of the Group B Streptococcus hemolytic lipid, Granadaene. *Frontiers in Microbiology* **10, 3123.**

Bastek, J. A., Gómez, L. M. and Elovitz, M. A. (2011). The role of inflammation and infection in preterm birth. *Clinics in Perinatology* **38(3), 385-406.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Berner, R. (2021). Group B Streptococcus vaccines: One step further. *The Lancet Infectious Diseases* **21(2), 158-160.**

Bianchi-Jassir, F., Seale, A. C., Kohli-Lynch, M., Lawn, J. E., Baker, C. J., Bartlett, L., Cutland, C., Gravett, M. G., Heath, P. T., Ip, M., Le Doare, K., Madhi, S. A., Saha, S. K., Schrag, S., Meulen, A. S., Vekemans, J. and Rubens, C. E. (2017). Preterm birth associated with Group B Streptococcus maternal colonization worldwide: Systematic review and meta-analyses. *Clinical Infectious Diseases* **65(Suppl 2), S133-S142.**

Boldenow, E., Gendrin, C., Ngo, L., Bierle, C., Vornhagen, J., Coleman, M. *et al.* (2016). Group B Streptococcus circumvents neutrophils and neutrophil extracellular traps during amniotic cavity invasion and preterm labor. *Science Immunology* **1(4), eaah4576.**

Buscetta, M., Papasergi, S., Firon, A., Pietrocola, G., Biondo, C., Mancuso, G., Midiri, A., Romeo, L., Teti, G., Speziale, P., Trieu-Cuot, P. and Beninati, C. (2014). FbsC, a novel fibrinogen-binding protein, promotes *Streptococcus agalactiae*-host cell interactions. *Journal of Biological Chemistry* **289(30), 21003-21015.**

Chan, G. J., Lee, A. C., Baqui, A. H., Tan, J. and Black, R. E. (2015). Prevalence of early-onset neonatal infection among newborns of mothers with bacterial infection or colonization: A systematic review and meta-analysis. *BMC Infectious Diseases* **15, 118.**

Coleman, M., Armistead, B., Orvis, A., Quach, P., Brokaw, A., Gendrin, C., Sharma, K., Ogle, J., Merillat, S., Dacanay, M., Wu, T., Munson, J., Baldessari, A., Vornhagen, J., Furuta, A., Nguyen, S., Adams Waldorf, K. M. and Rajagopal, L. (2021). Hyaluronidase impairs neutrophil function and promotes Group B streptococcus invasion and preterm labor in non-human primates. *mBio* **12(1), e03115-20.**

Cook, L. C. C., Hu, H., Maienschein-Cline, M. and Federle, M. J. (2018). A vaginal tract signal detected by the Group B streptococcus SaeRS system elicits transcriptomic changes and enhances murine colonization. *Infection and Immunity* **86(4), e00762-17.**

Edwards, J. M., Watson, N., Focht, C., Wynn, C., Todd, C. A., Walter, E. B., Phillips Heine, R. and Swamy, G. K. (2019). Group B Streptococcus (GBS) colonization and disease among pregnant women: A historical cohort study. *Infectious Diseases in Obstetrics and Gynecology* **2019, Article ID 5430493.**

Fallacara, A., Baldini, E., Manfredini, S. and Vertuani, S. (2018). Hyaluronic acid in the third millennium. *Polymers* **10(7), 701.**

Florindo, C., Ferreira, R., Borges, V., Spellerberg, B., Gomes, J. P. and Borrego, M. J. (2012). Selection of reference genes for real-time expression studies in *Streptococcus agalactiae*. *Journal of Microbiological Methods* **90(3), 220-227.**

Goldenberg, R. L., Hauth, J. C. and Andrews, W. W. (2000). Intrauterine infection and preterm delivery. *New England Journal of Medicine* **342, 1500-1507.**

Hahn, B. A., de Gier, B., van Kassel, M. N., Bijlsma, M. W., van Leeuwen, E., Wouters, M. G. A. J., van der Ende, A., van de Beek, D., Wallinga, J., Hahné, S. J. M. and van Hoek, A. J. (2021). Cost-effectiveness of maternal immunization against neonatal invasive Group B *Streptococcus* in the Netherlands. *Vaccine* **39(21), 2876-2885.**

Jeganathan, R. and Karalasingam, S. D. (2021). National Obstetrics Registry, 6th Report (2018-2020). National Obstetrics Registry, Malaysia.

Jiang, S. and Wessels, M. R. (2014). BsaB, a novel adherence factor of group B Streptococcus. *Infection and Immunity* **82(3), 1007-1016.**

Kayansamruaj, P., Pirarat, N., Katagiri, T., Hirono, I. and Rodkhum, C. (2014). Molecular characterization and virulence gene profiling of pathogenic *Streptococcus agalactiae* populations from tilapia (*Oreochromis* sp.) farms in Thailand. *Journal of Veterinary Diagnostic Investigation* **26(4), 488-495.**

Kolar, S. L., Kyme, P., Tseng, C. W., Soliman, A., Kaplan, A., Liang, J., Nizet, V., Jiang, D., Murali, R., Arditi, M., Underhill, D. M. and Liu, G. Y. (2015). Group B Streptococcus evades host immunity by degrading hyaluronan. *Cell Host and Microbe* **18(6), 694-704.**

Lin, F. C., Philips III, J. B., Azimi, P. H., Weisman, L. E., Clark, P., Rhoads, G. G., Regan, J., Concepcion, N. F., Frasch, C. E., Troendle, J., Brenner, R. A., Gray, B. M., Bhushan, R., Fitzgerald, G., Moyer, P. and Clemens, J. D. (2001). Level of maternal antibody required to protect neonates against early-onset disease caused by group B Streptococcus type Ia: A multicenter, seroepidemiology study. *Journal of Infectious Diseases* **184(8), 1022-1028.**

Liu, G., Zhang, W., Liu, Y., Yao, H., Lu, C. and Xu, P. (2014). Identification of a virulence-related surface protein XF in piscine *Streptococcus agalactiae* by pre-absorbed immunoproteomics. *BMC Veterinary Research* **10, 259.**

Liu, G. Y., Doran, K. S., Lawrence, T., Turkson, N., Puliti, M., Tissi, L. and Nizet, V. (2004). Sword and shield: Linked group B streptococcal β-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. *Proceedings of the National Academy of Sciences of the United States of America* **101(40), 14491-14496.**

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25(4), 402-408.**

Mu, R., Kim, B. J., Paco, C., Del Rosario, Y., Courtney, H. S. and Doran, K. S. (2014). Identification of a group B streptococcal fibronectin binding protein, SfbA, that contributes to invasion of brain endothelium and development of meningitis. *Infection and Immunity* **82(6), 2276-2286.**

Nanduri, S. A., Petit, S., Smelser, C., Apostol, M., Alden, N. B., Harrison, L. H., Lynfield, R., Vagnone, P. S., Burzlaff, K., Spina, N. L., Dufort, E. M.,

Schaffner, W., Thomas, A. R., Farley, M. M., Jain, J. H., Pondo, T., McGee, L., Beall, B. W. and Schrag, S. J. (2019). Epidemiology of Invasive early-onset and late-onset group B Streptococcal disease in the United States, 2006 to 2015: Multistate laboratory and population-based surveillance. *JAMA Pediatrics* **173(3), 224-233.**

Nogacka, A., Salazar, N., Suárez, M., Milani, C., Arboleya, S., Solís, G., Fernández, N., Alaez, L., Hernández-Barranco, A. M., de Los Reyes-Gavilán, C. G., Ventura, M. and Gueimonde, M. (2017). Impact of intrapartum antimicrobial prophylaxis upon the intestinal microbiota and the prevalence of antibiotic resistance genes in vaginally delivered full-term neonates. *Microbiome* **5(1), 93.**

Otaguiri, E. S., Morguette, A. E. B., Tavares, E. R., dos Santos, P. M. C., Morey, A. T., Cardoso, J. D., Perugini, M. R. E., Yamauchi, L. M. and Yamada-Ogatta, S. F. (2013). Commensal *Streptococcus agalactiae* isolated from patients seen at University Hospital of Londrina, Paraná, Brazil: Capsular types, genotyping, antimicrobial susceptibility and virulence determinants. *BMC Microbiology* **13, 297.**

Rajagopal, L., Vo, A., Silvestroni, A. and Rubens, C. E. (2006). Regulation of cytotoxin expression by converging eukaryotic-type and two-component signalling mechanisms in *Streptococcus agalactiae*. *Molecular Microbiology* **62(4), 941-957.**

Randis, T. M., Gelber, S. E., Hooven, T. A., Abellar, R. G., Akabas, L. H., Lewis, E. L., Walker, L. B., Byland, L. M., Nizet, V. and Ratner, A. J. (2014). Group B Streptococcus β-hemolysin/cytolysin breaches maternal-fetal barriers to cause preterm birth and intrauterine fetal demise *in vivo*. *Journal of Infectious Diseases* **210(2), 265-273.**

Russell, N. J., Seale, A. C., O'Sullivan, C., Le Doare, K., Heath, P. T., Lawn, J. E., Bartlett, L., Cutland, C., Gravett, M., Ip, M., Madhi, S. A., Rubens, C. E., Saha, S. K., Schrag, S., Meulen, A. S., Vekemans, J. and Baker, C. J. (2017). Risk of early-onset neonatal group B streptococcal disease with maternal colonization worldwide: Systematic review and meta-analyses. *Clinical Infectious Diseases* **65(2), 152-159.**

Santi, I., Maione, D., Galeotti, C. L., Grandi, G., Telford, J. L. and Soriani, M. (2009). BibA induces opsonizing antibodies conferring *in vivo* protection against group B streptococcus. *Journal of Infectious Diseases* **200(4), 564-570.**

Schrag, S. J. and Verani, J. R. (2013). Intrapartum antibiotic prophylaxis for the prevention of perinatal group B streptococcal disease: Experience in the United States and implications for a potential group B streptococcal vaccine. *Vaccine* **31(4), 20-26.**

Seale, A. C., Bianchi-Jassir, F., Russell, N. J., Kohli-Lynch, M., Tann, C. J., Hall, J. *et al.* (2017). Estimates of the burden of Group B Streptococcal disease worldwide for pregnant women, stillbirths, and children. *Clinical Infectious Diseases* **65(2), 200-219.**

Seo, H. S., Minasov, G., Seepersaud, R., Doran, K. S., Dubrovska, I., Shuvalova, L., Anderson, W. F., Iverson, T. M. and Sullam, P. M. (2013). Characterization of fibrinogen binding by glycoproteins Srr1 and Srr2 of *Streptococcus agalactiae*. *Journal of Biological Chemistry* **288(50), 35982-35996.**

Sheen, T. R., Jimenez, A., Wang, N. Y., Banerjee, A., van Sorge, N. M. and Doran, K. S. (2011). Serine-rich repeat proteins and pili promote *Streptococcus agalactiae* colonization of the vaginal tract. *Journal of Bacteriology* **193(24), 6834-6842.**

Stern, R. and Jedrzejas, M. J. (2006). Hyaluronidases: Their genomics, structures, and mechanisms of action. *Chemical Reviews* **106(3), 818-839.**

Tapiainen, T., Koivusaari, P., Brinkac, L., Lorenzi, H. A., Salo, J., Renko, M., Pruikkonen, H., Pokka, T., Li, W., Nelson, K., Pirttilä, A. M. and Tejesvi, M. V. (2019). Impact of intrapartum and postnatal antibiotics on the gut microbiome and emergence of antimicrobial resistance in infants. *Scientific Reports* **9, 10635.**

Vekemans, J., Moorthy, V., Friede, M., Alderson, M. R., Meulen, A. S., Baker, C. J., Heath, P. T., Madhi, S. A., Doare, K. M., Saha, S. K., Schrag, S. and Kaslow, D. C. (2019). Maternal immunization against Group B streptococcus: World Health Organization research and development technological roadmap and preferred product characteristics. *Vaccine* **37(50), 7391-7393.**

Verani, J. R., McGee, L. and Schrag, S. J. (2010). Prevention of perinatal group B streptococcal disease: Revised guidelines from CDC, 2010. *Recommendations and Reports: Morbidity and Mortality Weekly Report* **59(RR-10), 1-36.**

Vogel, J. P., Chawanpaiboon, S., Moller, A., Watananirun, K., Bonet, M. and Lumbiganon, P. (2018). The global epidemiology of preterm birth. *Best Practice and Research: Clinical Obstetrics and Gynaecology* **52, 3-12.**

Vornhagen, J., Quach, P., Boldenow, E., Merillat, S., Whidbey, C., Ngo, L. Y., Adams Waldorf, K. M. and Rajagopal, L. (2016). Bacterial hyaluronidase promotes ascending GBS infection and preterm birth. *mBio* **7(3), e00781-16.**

Wang, N. Y., Patras, K. A., Seo, H. S., Cavaco, C. K., Rösler, B., Neely, M. N., Sullam, P. M. and Doran, K. S. (2014a). Group B streptococcal serine-rich repeat proteins promote interaction with fibrinogen and vaginal colonization. *Journal of Infectious Diseases* **210(6), 982-991.**

Wang, Z., Guo, C., Xu, Y., Liu, G., Lu, C. and Liu, Y. (2014b). Two novel functions of hyaluronidase from *Streptococcus agalactiae* are enhanced intracellular survival and inhibition of proinflammatory cytokine expression. *Infection and Immunity* **82(6), 2615-2625.**

Whidbey, C., Harrell, M. I., Burnside, K., Ngo, L., Becraft, A. K., Iyer, L. M., Aravind, L., Hitti, J., Adams Waldorf, K. M. and Rajagopal, L. (2013). A hemolytic pigment of Group B Streptococcus allows bacterial penetration of human placenta. *Journal of Experimental Medicine* **210(6), 1265-1281.**