



## VIRULENCE PROFILE OF BULGARIAN CLINICAL ISOLATES *STREPTOCOCCUS AGALACTIAE*- PCR DETERMINATION

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### Abstract

**Purpose:** This study aims to determine the virulence profile of the circulating Bulgarian clinical isolates of GBS including the highly virulent serotype III by molecular methods and evaluate the differences among studied isolates from healthy pregnant women and non-pregnant ill persons.

**Material and methods:** We investigated 104 non-duplicate isolates of GBS obtained from pregnant (n=15) and non-pregnant women (n=89), living in Sofia, Bulgaria. All GBS strains were screened for the presence of important virulence genes, namely *bca*, *cyl(E)*, *rib*, *hyl(B)* and the capsular serotype III detected by Real-time PCR.

**Results:** PCR detection of five important virulence genes showed that *cylE* (91.3%) was the most prevalent one among the total isolates of GBS. Genes encoding the C protein (*bca*), hyaluronate lyase (*hylB*), Alp family protein Rib (*rib*) were presented in 51%, 17.3% and 22.1% respectively of the examined isolates. By using real-time PCR, we found that 13.5% from all tested Bulgarian GBS strains confirmed as GBS serotype III and harbored *cpsI* gene. No significant differences between the two groups were observed excluding the presence of *cpsI*.

**Conclusion:** The risk of neonatal and prenatal GBS infection in 1/3 of healthy pregnant women colonized by streptococcal strains with highly virulent capsular serotype in combination with other strong virulent factors was demonstrated by this study. The regular screening for GBS and properly treatment could be reduce the severe maternal-fetal infections due to this microorganism.

**Keywords:** *Streptococcus agalactiae*, PCR, virulence factors,

### 1. INTRODUCTION

*Streptococcus agalactiae* (group B *Streptococcus* - GBS) is often isolated from the lower gastrointestinal and genital tract of healthy adults. It is also a leading cause of neonatal morbidity and mortality, it is an important etiological agent of severe infectious diseases such as neonatal meningitis, sepsis, those in patients with diabetes mellitus or compromised immune defence and orthopedic device infections [1-3]. Various pathogen and host factors mediate specific host-cell interactions and in-

terfere with innate immune clearance mechanisms. GBS has many virulence factors including adhesins like C5a peptidase (ScpB), laminin-binding protein (Lmb), the  $\alpha$  and  $\beta$ -subunits of C protein (Bca and Bac), Rib protein and produces a variety of toxins (invasins) such as  $\beta$ -hemolysin/cytolysin (*cylE*), hyaluronidase (*hylE*) and the CAMP factor (*cfb*) to promote bacterial entry and survival within host cells [2, 4]. The GBS capsular polysaccharide (CPS) plays a critical role against effective opsono-phagocytic killing [2, 5]. To date, on the basis of the antigenic properties of CPS this species can be classified into 10 different serotypes (Ia, Ib, II-IX) [5, 6]. Plurality studies suggested that the CPS of type III GBS is a major virulence factor of this organism, which significantly appeared to be associated with invasive neonatal disease and almost all meningitis, defined as highly virulent clones such as ST-17 [7-10]. Molecular serotyping of GBS based on the detection of serotype-specific genes of the capsular region has been developing rapidly [11].

This study aims to determine the virulence profile of the circulating Bulgarian clinical isolates of GBS, including the highly virulent serotype III by molecular methods and evaluate the differences among studied isolates from healthy pregnant women and non-pregnant ill persons.

### 2. MATERIALS AND METHODS

#### 2.1. GBS strains.

A total of 104 non-duplicate isolates of GBS were obtained from pregnant (n=15) and non-pregnant women (n=89), living in Sofia, Bulgaria, aged 20 - 59 years during the period of September 2018 to May 2019. The patients were divided into two groups: I. Group - Pregnant women were asymptomatic colonized; while II. Group - non-pregnant persons were with clinical manifestations of vaginitis or cystitis. The clinical isolates were acquired from vaginal samples (n=91) and samples of urine (n=13).

The identification of the suspected bacterial isolates was accomplished under routine criteria, using beta-hemolysis on blood agar, a catalase-negative reaction, a positive CAMP test, a lack of susceptibility to bacitracin and a negative pyrrolidonyl arylamidase test (PYR) for presumptive detection. For determining of Lancefield serological groups, a PathoDextra Strep Grouping Kit

Thermo Scientific (Oxoid, UK) was used. For definitive biochemical identification, we used Crystal GP (Becton Dickinson, Kelberg, Germany) when needed. GBS strains were stored in skim milk at -70°C and were sub-cultivated three times on Columbia agar with 5% sheep blood (Becton Dickinson, Kelberg, Germany) before the experimental work.

## 2.2. Extraction of DNA.

DNA extraction was performed using a DNA sorb AM nucleic acid extraction kit (AmpliSens, Russia), according to the manufacturer's guidelines. For the purpose of DNA extraction, GBS strains were cultured on Columbia blood agar (Becton Dickinson, Kelberg, Germany) for 24 h at 35°C in an atmosphere with 5% CO<sub>2</sub>.

## 2.3. Polymerase chain reaction (PCR) determination of virulence genes of GBS

All GBS strains were screened by PCR for the presence of important virulence genes, namely *Bca* (encoding beta Subunit of the C protein), *cyl(E)* (encoding cytolysin-heamolysin), *rib* (Alp family protein Rib), *hyl* (B) (encoding hyaluronidase protein), using previously

described specific primers [4,5]. PCR cycling conditions and primers sequences were presented in table 1.

## 2.4. Real-time PCR determination of highly virulent type III GBS

Detection of the capsular serotype III was carried out by using primers designed to amplify a unique region of the polysaccharide capsular gene *cpsI*, described previously (13). Reaction mixtures consisted 6.25 µl of Universal Master Mix (Applied Biosystems, Darmstadt, Germany) containing an AmpliTaq Gold DNA polymerase, dNUTPs, MgCl<sub>2</sub> and reaction buffer, 100 nM of fluorescence-labeled TaqMan probe, 1 µl of purified template DNA and using 300 nM of each primer in final volume 25 µl. DNA was amplified at following conditions: Initial denaturation 95°C for 3 min; Denaturation 95°C - 30 sec; Annealing temperature 58°C - 40 sec; Final elongation 72°C - 1 min, using Line-Gene K machine (Bioer Technology Co., LTD., Bio Flux, Corporation, Tokoy, Japan). All positive samples were confirmed by conventional PCR with expected size of the amplicons 170bp (Tabl. 1).

**Table 1.** PCR cycling conditions and primers sequences used in the study.

Target genes	Sequence (Forward 5' → 3'), (Reverse 5' → 3')	Melting temperature	Amplicon Size (bp)
<i>cylE</i>	F- TGACATTTACAAGTGACGAAG R- TTGCCAGGAGGAGAATAGGA	54 °C	300
<i>Bca</i>	F- TAACAGTTATGATACTTCACAGAC R- ACGACTTTCTTCCGTCCTTAGG	54 °C	535
<i>Rib</i>	F- CAGATGCCGATAAGA R- TACGCGGATCGACAA	52 °C	237
<i>hylB</i>	F- CCGTTATCAGTTACAGGTC R- GTCGATGTAAGAACCGTCAGC	54 °C	714
<i>cpsI</i>	F- GGAATTGTTCTTTATTTTCTGCCT R- ACTATACCAAAGTTGAGAATA ATAATACAATACTCCAATGA	58 °C	170

## Statistical Analysis

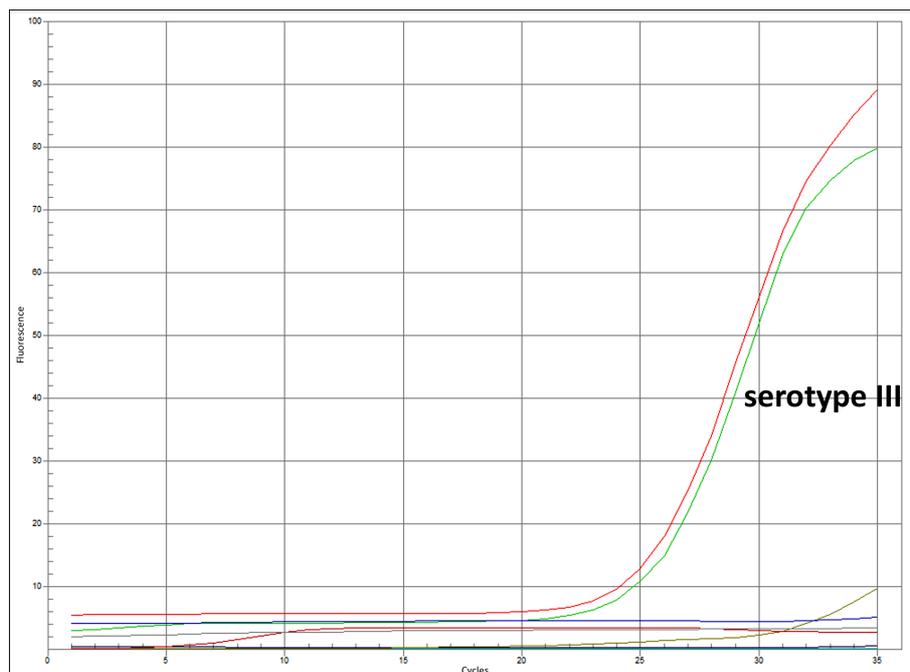
Differences were analyzed using unpaired Descriptive statistics, Fisher's exact test, SPSS for Windows, Version 19.0 USA, Chicago, SPSS Inc. The significance of results was determined at the level of  $p \leq 0.05$ .

## RESULTS

PCR detection of five important virulence genes showed that *cylE* (91.3%) was the most prevalent one among the total isolates of GBS. Genes encoding the C protein (*bca*), hyaluronate lyase (*hylB*), Alp family pro-

tein Rib (*rib*) were presented in 51%, 17.3% and 22.1% respectively of the examined isolates, showed in table 2. By using real-time PCR, we found that 13.5% (n=14) from all tested Bulgarian GBS strains confirmed as GBS serotype III and harbored *cpsI* gene. Some of the the real-time PCR results were shown fig.1. The distribution of genetic elements encoding virulence factors among 104 isolates of GBS from two groups patients were presented in Table 2. No significant differences between the two groups were observed excluding the presence of *cpsI*.

**Fig. 1.** Real-time PCR-based detection of serotype III in Bulgarian GBS strains.



**Table 2.** Distribution of virulence factors among 104 isolates of GBS from two groups patients.

	Non-preganant women n= 89		Pregnant women n=15		Total n=104		P value
	N	%	n	%	n	%	
<i>cylE</i>	81	<b>91</b>	14	<b>93.3</b>	95	<b>91.3</b>	<b>1.000</b>
<i>Bca</i>	46	<b>51.7</b>	7	<b>46.7</b>	53	<b>51</b>	<b>0.7849</b>
<i>Rib</i>	19	<b>21,3</b>	4	<b>26,7</b>	23	<b>22,1</b>	<b>0.7375</b>
<i>hylB</i>	16	<b>18</b>	2	<b>13,3</b>	18	<b>17,3</b>	<b>1.000</b>
<i>cpsI</i>	9	<b>10,1</b>	5	<b>33.3</b>	14	<b>13,5</b>	<b>0.0291</b>

\*The underlined text shows a statistically significant difference

### DISCUSSION

GBS can lead the development of the various pathogenic mechanisms due to the fact that it expresses a large number of virulence factors that allow it to suppress the host's immune system. In the present work, the most frequently found virulence factor was *cyl* (*E*) in 91.3%. There are some data about this gene encoding GBS  $\beta$ -haemolysin/cytolysin promotes lung epithelial cell invasion and triggers the release of interleukin-8 (IL-8), a principal neutrophil chemoattractant [2]. Other authors reported that moreover,  $\beta$ -haemolysin / cytolysin toxin contributes directly to cardiomyocyte dysfunction and apoptosis, which may increase its role in pathophysiological anomalies of GBS sepsis [13]. The gene encoding the alpha antigen, *bca*, was found in 1/2 of the circulating isolates of GBS in our region. *Bca* is homologous to the antiphagocytic M-proteins of group A streptococci, and the antigenicity and virulence of GBSstrains containing

the alpha antigen is depend on the number of repeats expressed [14]. Our results for the finding if this gene are similar to those from Spain [15], but not in those from China, where *bca* is found in only 21% from studying GBS strains [16]. In our study, *hyl*(*B*) and *rib* gene were detected in 17.3%, resp. 22.1% from the examined streptococcal isolates. It is a higher rate for *hyl*(*B*) than this one from Spain [17], and low finding for *rib* in comparing to the reports from Spain, China, Iran [15-17]. The present results showed that 13.5% of Bulgarian GBS strains belong to the highly virulent serotype III, encoded by *cpsI*, which cause most cases of neonatal meningitis [8-10]. In general many studies report that serotype III GBS is predominant in most part of the world [18, 19]. Compared to an overall serotype III prevalence of 25%, Central America and South-Eastern Asia, as well as some South Asian countries (11%) had a lower reported prevalence of serotype III [19]. Our study showed that Bulgar-

ian isolates had a prevalence of GBS serotype III in the group of pregnant women. A recent Serbian report showed that CPS type III predominated in both invasive and non-invasive study groups [20].

The streptococcal isolates from healthy pregnant women of GBS and those from ill non-pregnant patients were with no significant difference in the rate of genes in their virulence profile excluding the presence of *cpsI*, that showed higher frequencies in pregnant ones. In the previous study, other Bulgarian authors showed that early-onset neonatal GBS infection in 77.3% of the cases came with clinical symptoms of Maternal-fetal infection [21]. The present results demonstrated that the Bulgarian clini-

cal isolates GBS harbored many virulence determinants and especially the finding of *cpsI*, encoding highly virulent serotype III predominantly in 33.3% pregnant healthy carriers.

## CONCLUSION

The risk of neonatal and prenatal GBS infection in 1/3 of healthy pregnant women colonized by streptococcal strains with highly virulent capsular serotype in combination with other strong virulent factors was demonstrated by this study. The regular screening for GBS and properly treatment could be reduce the severe maternal-fetal infections due to this microorganism.

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