Antibiotic resistance, biofilm formation and virulence genes of Streptococcus agalactiae serotypes of Indian origin

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Abstract

Introduction:

Streptococci are diverse group of gram-positive bacteria which causes serious life-threatening infections in developed as well as developing nations. Group B Streptococcus (GBS) or *Streptococci agalactiae* is one of the serologically distinct species of genus streptococcus which colonizes genitourinary and lower gastrointestinal tracts of female populations. GBS is a causative agent of various infections in new-born, non-pregnant adults and pregnant women. Understanding antibiotic resistance profiling will provide insight into the development of antibiotic prophylaxis against GBS infection. The aim of this study was to determine biofilm forming ability, presence of virulence genes and antibiotic susceptibility pattern of GBS clinical isolates.

Methods:

Previously, identified 30 GBS isolates were used to check antibiotic susceptibility and resistance by Kirby-Bauer disk-diffusion method. All GBS isolates were subjected to biofilm detection using the Congo Red Agar (CRA) and Crystal Violet Assay (CVA). GBS isolates were subjected to PCR for the detection of various virulence genes involved in adhesion and invasion.

Results:

Resistance towards penicillin was observed to be 26.6% (n=8 strains) as compared to other tested antibiotics which indicates the emergence of penicillin resistance among GBS isolates. Susceptibility for ofloxacin as 93.33% (n=28) was found to be highest, followed by azithromycin as it exhibited a 90% (n=27) susceptibility rate. Majority of the GBS isolates were found to be strong biofilm producers 70% (n=21) and 30% (n=9) were found to be moderate biofilm producers.

Conclusions:

We demonstrated, GBS isolates exhibited resistance towards penicillin and a negative correlation between biofilm formation and penicillin was found. Further, we found, both strong and moderate biofilm producing isolates have most of the virulence genes at genetic level, however, strong biofilm formation phenotype was not significantly associated with the expression of any virulence gene.

Introduction

Streptococcus agalactiae, also known as Group B Streptococcus (GBS), is a gram positive opportunistic pathogen which causes severe infections such as sepsis, pneumonia and meningitis in neonates [1, 2]. Based on their capsular polysaccharide composition, ten serotypes of GBS have been reported (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX). Amongst serotypes Ia, Ib, II, III, and V are the most commonly isolated serotypes [3–5]. In India, serotype Ia and III were found to be the most prevalent serotypes [6]. It has been reported that GBS can form aggregates on interfaces, called biofilms that allowed them to survive in hostile

environments and provide protection against antibiotics [7]. However, the connection between biofilm and antibiotic resistance is of considerable interest as biofilm-mediated resistance in several pathogens including GBS [8, 9]. It has been reported that approximate 65% of microbial infections are associated with biofilms and are ubiquitously presenting a significant role in many persistent infections [10]. Notably, it has been demonstrated that low doses of certain antibiotics induce biofilm formation indicating that biofilm development may be involved in the global response to external stresses, including antibiotics [11]. In recent years, the ability of GBS to form biofilms has attained significant attention for its possible role in survival and pathogenesis [12]. It has been observed that biofilm formation in GBS not only enhance its ability to colonize but also helps in survival in host [13]. Importantly, biofilm formation in GBS also increases antibiotic resistance by expressing specific genes [14].

Upon infection, virulence factors aid the bacteria to adapt to the changing host environments and provide survival strategies including biofilm formation that facilitates the disease manifestation [15]. In this regard, GBS expresses a diverse array of surface-associated and secreted virulence factors that mediate specific host-cell interactions and interfere with innate immune clearance mechanisms. Some of the virulence factors have been identified and characterized which include; adhesion and invasion factors that assist the bacteria to colonize the both epithelial and endothelial tissues and invade across the host barrier. Evasion factors, decrease neutrophil recruitment and intercept the complement binding, pore-forming toxins that damage the host cells, and factors that repel or otherwise induce resistance to antimicrobial peptides [16, 17]. The ability of GBS infections possibly is determined by various virulence genes of GBS, such as *gbs67, cylE, cfb, scpB, lmb*, and *pavA*, which encodes for an ancillary protein of pili, β-hemolysin, Christie-Atkins-Munch-Peterson (CAMP), surface enzyme scpB (C5a peptidase), laminin-binding protein, aggregation factor, respectively [18].

To best of our knowledge, no information is available related to the role of biofilm formation and antibiotic resistance in GBS serotypes of Indian origin. In the current study, we have examined the mechanism of biofilm formation in various GBS clinical isolates of Indian origin and studied a correlation between biofilm formation and antibiotic resistance. In addition, we have also analyzed the distribution pattern of different selected virulence genes to determine correlation between biofilm formation and virulence genes.

Materials And Method

GBS serotypes and culture conditions

A total of 30 clinical GBS isolates collected previously [6] were used in this study. All these strains were inoculated in Todd Hewitt broth medium (THB; Himedia Laboratories, India) and were stored in 25% glycerol at -80^{o}C for further use. Briefly, cultures were streaked from frozen stock on 0.5 % sheep blood agar plate (BAP) with the help of sterile loop and incubated at 37^{o}C for 24-30 hrs.

Antibiotic susceptibility test

To determine the antibiotic resistance profile, all the 30 GBS clinical isolates were screened against six antibiotics according to the Clinical and Laboratory Standards Institute Guidelines [19]. The Kirby-Bauer disk-diffusion method was used to test penicillin (0.06µg), clindamycin (2µg), erythromycin (15µg), gentamicin (10µg), ofloxacin (2µg) and azithromycin (15µg) resistance. Briefly, fresh sheep blood agar plates were inoculated with homogeneous GBS suspension of 0.5 McFarland standards, prepared from fresh bacterial cultures according to the CLSI [20]. 0.5 McFarland standards were used as reference with an optical density comparable to 1.5 x 10^8 bacterial colony forming units (CFU/ml).

Pure cultures were streaked on Todd Hewitt broth (THB) agar plates with 1 % glucose and incubated at 37°C for overnight. Single purified colony was picked and mixed into phosphate buffer saline (1X), and used to form a bacterial lawn on fresh 5 % sheep blood agar plate (BAP) (Life Science Media, Delhi India). Specific antibiotic discs were placed onto the plate using sterile forceps and were incubated overnight at 37°C. The zone of inhibition was measured using mathematical scale manually and the results were interpreted as susceptible, intermediate, and resistant according to the Clinical and Laboratory Standard Institute Guidelines (CLSI) [19].

Biofilm formation assay

Biofilm formation of GBS clinical isolates was assessed by Congo Red Agar (CRA) and Crystal Violet Assay (CVA) as described [21]. CRA is a qualitative assay whereas, CVA is a quantitative method for biofilm detection. In brief, THB medium supplemented with 1% glucose and 0.08% CRA plates was used to assess the biofilm formation phenotype. Briefly, CRA plates were inoculated with GBS cell suspension and incubated at 37°C for 24 hours. After overnight incubation, isolates were interpreted according to their colony phenotypes and change in color. The formation of black colored colonies with slime production was used as an indicator for biofilm formation [22].

In case of CVA, 5ml THB broth with 1% glucose was inoculated with single isolated colony from the fresh blood agar plate and incubated at 37°C until their OD_{600} reaches between 0.4-0.6. At desired OD_{600} (~ 0.5), 100µL culture was dispensed in 96-well microtiter plate along with 100µL fresh THB with 1% glucose and incubated at 37°C for 48 hours under static condition. After incubation plates were gently washed 3 times with 1X PBS (pH 7.4) followed by heat fixation at 60°C for 1 hour and was stained with 100µL of 0.5% crystal violet (CV) for 5 minutes. After CV staining, plates were washed three times with 1X PBS and the remaining CV was solubilized by addition of 200µL of 95% ethanol, and plates were incubated at room temperature for 10 minutes. The optical density of each well was measured by the ELISA plate reader at 595nm (BioTek Synergy™ H1 hybrid multi-mode microplate reader, USA). OD values less than 2 was considered as non-biofilm producers, 2-4 as weak biofilm producers, 4-8 as moderate biofilm producers and more than 8 as strong biofilm producers [18]. *Pseudomonas aeruginosa* (MTCC 2297) and THB broth +1% glucose (only medium) was used as positive and negative controls, respectively [23]. All experiments were performed in triplicate with three independent experiments.

Detection of virulence genes by PCR

Six virulence genes (*gbs67, cylE, cfb, scpB, lmb, and pavA*) were targeted their expression and role in biofilm formation using semi-quantitative PCR using gene specific primer (**Supplementary Table 1)**. For this, genomic DNA was isolated by CTAB (Cetyltrimethylammonium bromide) method [24]. In brief, 50 ml of log phase GBS culture (OD_{600} 0.5) in THB was centrifuged at 6000 rpm for 5 min at room temperature. Pellet, obtained was washed twice with 5 ml of 0.1M tris buffer (pH 8.0). To this, 50 µl of lysozyme (100mg/ml), 200 µl of 10% SDS and 60 µl of proteinase K (60mg/ml) were added and this mixture was incubated at 65^{o}C for 2 h. Further, 500 µl of 5M NaCl and 800 µl of 10% CTAB was added and mixed gently and incubated for 30 min at 65^{o}C. After incubation, 15ml of chloroform: isoamylalcohol (24:1) was added and vortexed for 10 sec and centrifuged at 12,000 rpm for 10 min. Upper aqueous phase was collected and an equal volume of phenol: chloroform (1:1) was added to it. After vortexing for 5 sec, and centrifugation at 12,000 rpm for 5 min, upper aqueous phase was collected and 2 µl of RNAse (10mg/ml) was added followed by incubation at 37^{o}C. After 30 min, equal volume of chilled absolute ethanol was added and incubated for 2 hrs at -20^{o}C and centrifuged at 12,000 rpm for 10 min. Supernatant was discarded and the pellet obtained was washed with chilled 70% ethanol (1 ml) and centrifuged for 15 min at 12,000 rpm at 4^{o}C. After centrifugation DNA pellet was dissolved in nuclease free water. Quantification and purity of extracted genomic DNA was checked by measuring the absorbance at 260nm and 280nm on UV spectrophotometer. The PCR reaction contains 2mM $MgCl_2$, 5pmole of each forward and reverse primer,0.5mM dNTPs mix, 100ng genomic DNA template, 2 units high-fidelity DNA Polymerase. The PCR conditions used were as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 45 s, 40–57°C (depending on primer melting temperature) for 45 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCR amplified DNA was resolved on 1.2% TAE agarose gel and visualized under UV light. The gyrase subunit A(*gyrA*)gene was used as an internal standard [25].

Results

Antibiotic susceptibility

For all tested antibiotics, most of the GBS isolates were found to be more resistant to penicillin (26.6%; n=8 strains) as compared to other tested antibiotics which indicates the emergence of penicillin resistance among GBS isolates. In case of erythromycin and clindamycin, resistance rate was found to be 10% (n=3), followed by azithromycin, 6.67% (n=2). In case of gentamicin and ofloxacin, GBS isolates showed 3.33% resistance (n=1).

Among all antibiotics tested, susceptibility was found to be highest in case of ofloxacin as 93.33% (n=28) strains were found to be susceptible, followed by azithromycin as it exhibited a 90% (n=27) susceptibility rate. In case of clindamycin susceptibility rate was 76.67% (n=23) followed by penicillin 73.33 % (n=22) and gentamicin 66.67% (n=20) (**Figure 1**). These data altogether suggest that 33.33% of GBS isolates were susceptible to all antibiotics and 66.67% (n=20) were either resistant or intermediate to at least one antibiotic. Specifically, 11 GBS isolates out of 30, were resistant to at least one antibiotic.

Detection of the biofilm-producing phenotype

CRA was used to determine biofilm-producing phenotype as production of extracellular polymeric substances (EPS) by GBS isolates. Change in the color of media from red to black was used as an indicator in order to designate GBS as a biofilm producer. In case of CVA, 70% (n=21) of GBS isolates and 30% (n=9) were found to be strong and moderate biofilm producers respectively **(Figure 2a)**. The biofilm-forming capacity of each isolate is summarized in **Figure (2b)**. Interestingly, no weak biofilm producer and non-biofilm producer isolates were found. Comparative growth rate studies showed that there was no significant difference in the growth rate of the strong biofilm producer and moderate biofilm producing GBS isolates (data not shown). GBS serotypes Ia, II and V were found to be strong biofilm producing serotypes as compared to serotype III and VII (**Figure 3**).

Correlation between biofilm formation and antibiotic resistant

To establish a correlation between biofilm formation and antibiotic resistance spearman's correlation coefficient (rs) was determined. A positive correlation among biofilm formation with individual antibiotics such as azithromycin (rs=0.2, P > 0.05), clindamycin (rs=0.126, P > 0.05), erythromycin (rs=0.269, P > 0.05), gentamicin (rs=0.188, P > 0.05) and ofloxacin (rs=0.289, P > 0.05) was observed. However, for penicillin, negative correlation was found which suggest, GBS isolates were less susceptible to penicillin and biofilm correlates with antibiotic resistance **(Figure 4)**.

Detection of virulence genes and their correlation with the biofilm

Previous studies have shown the prevalence of virulence genes in biofilm producing isolates than those in the non-producing isolates [26]. In case of uroptahogenic *Escherichia coli*, biofilm forming isolates were found to have more virulence genes as compared to non-biofilm producers [27]. Therefore, in the present study, correlation between virulence gene present and biofilm producing phenotype of GBS isolates were also assessed.The virulence genes identified were namely: *cylE* (96.67%), *pavA* (96.67%), *cfb* (93.33%), *lmb* (90%), *gbs67* (76.67%), and *scpB* (40%) **(Figure 5)**. Except for *scpB* there was no significant differences was found in virulence genes between serotype III and V **(Figure 6**). A high frequency of the virulence genes: *cfb, lmb, cylE, pavA,* and *gbs67* was detected in all clinical isolates of GBS and the majority of the isolates carried multiple virulence genes which exhibited GBS's ability to adhere, colonize, and invade host tissues.

A correlation between strong biofilm producer vs moderate biofilm producing strains with distribution of virulence genes was also determined. We hypothesized that whether presence of virulence gene (s) contributes to strong biofilm producing phenotype or not. We found that biofilm formation was not significantly associated with the expression of any virulence gene. These data altogether suggest that *cfb*, *lmb*, *cylE,* and *pavA* were present in 95% of strong biofilm producing isolates followed by *gbs67* (74%) and *scpB* (43%) whereas *cylE* and *pavA* were present in all moderate biofilm producing isolates (100%) followed by *cfb* (89%), *lmb* (78%), *gbs67* (67) and *scpB* (33). However, low occurrence of *scpB* was observed in both strong and moderate biofilm producing GBS isolates. (**Figure 7).**

Discussion

Recently exploitation of antibiotics has resulted in the emergence of antibiotic resistance in bacterial species which is a global concern [28]. Not only over use of antibiotics contributes to emergence of biofilm-mediated multi drug resistance strains, but microbes can also adapt themselves to different external stress conditions and form biofilm. It has been suggested that biofilm formation play important role in antibiotic resistance, which makes it hard to treat bacterial infection [29]. It has been reported that biofilm formation generate resistance towards antibiotics by decreasing the antibiotic penetration rate and mediating bacterial gene expression [30]. Biofilm also play a role in the pathogenesis. In this scenario bacterium residing in the biofilm can become resistant to the immune system, antibiotics, and other treatments [31]. Emergence of antibiotic resistance by GBS clinical isolates, emphasize the need for continuous monitoring of antimicrobial resistance patterns. A few studies have shown that the vast majority of GBS isolates developed resistance towards antibiotics against GBS infections. Sensitivity of 98% of GBS isolates towards penicillin and 78.3% and 88.3% of GBS towards erythromycin and clindamycin respectively have been recently reported [32].

GBS is considered as susceptible to beta-lactam antibiotics and some countries adopt an intrapartum prophylaxis regime to prevent the GBS infections. However, several reports of reduced susceptibility of GBS strains to beta-lactam antibiotics including penicillin have been observed [33–36]. In a study, resistance of GBS clinical strains isolated from pregnant women was reported to be highest for tetracycline (89.66%), followed by erythromycin (76.23%) and clindamycin (58.21%) [37]. Previous reports have shown GBS resistance towards macrolide antibiotics in Taiwan which indicates that resistance to macrolide is much higher than western countries and ranges from 11.5 to 32% [38–40]. Therefore, in the present study, we investigated antimicrobial activity of 6 widely used antibiotics against GBS clinical isolates of Indian origin. We found increased resistance towards penicillin among type III and V GBS isolates (invasive strains of GBS). In our study, we have found increased resistance of GBS clinical isolates towards penicillin, whereas in case of other tested antibiotics GBS isolates remain either intermediate or sensitive.

Several studies have shown that biofilm formation can be induced under the low doses of certain antibiotics indicating that antibiotics can result in the formation of biofilm under external stresses [11, 41]. In the cutrrent study we have observed that all GBS isolates were able to form biofilm. 70% of GBS isolates were strong biofilm producers whereas, 30% were assessed as weak biofilm producing GBS isolates. We have also observed that out of 30 GBS isolates, 33.33% were susceptible to all antibiotics tested in this study and 66.67% (n = 20) were either found to be resistant or intermediate to at least one antibiotic. Among 30 GBS strains, 78.9% were found to be susceptible, 11.1% were found to be intermediate and 10% have shown resistance to antibiotics tested (either one or two tested antibiotics, however, none of the isolates was found to be resistant for all antibiotics). We found all GBS isolates were capable to form strong and moderate biofilm. Previously, it has been reported that antibiotics resistance is achieved by biofilm development and both have an association with each other. Extensive use of antibiotics leads to acquisition of antibiotic resistance. In uropathogenic *E. coli* isolates, biofilm

forming isolates are highly associated with multi-drug resistance phenotype than the non-biofilm producers [27]. Hence, to find out if there is any possible relationship exists between antibiotic resistance and ability to form biofilm among the GBS isolates, we performed statistical analysis by using spearman's rank correlation test. In our study penicillin was the only antibiotic that exhibited association with the ability to form biofilm for GBS isolates as negative correlation was observed by spearman's correlation coefficient. However, in case of other tested antibiotics no such relationship was observed.

Antibiotic susceptibility data and biofilm data altogether suggest that those GBS isolates which produce strong biofilm are not susceptible to penicillin and the effect of penicillin decreases in these isolates. Further, it has been reported that virulence-associated genes are involved in pathogenicity by enhancing the biofilm formation which suggests an association between the presence and expression of virulence genes with biofilm production. Previous studies have demonstrated that a correlation exits between occurrences of virulence genes and biofilm formation [27], therefore, the relationship between biofilm formation and possibly related virulence factors of GBS isolates was determined in the present study. However, we have not observed any such correlation between occurrences of virulence genes with strong biofilm producing phenotype in GBS isolates. Since the association of antimicrobial resistant in GBS and biofilm formation has not been studied, this research was conducted to assess the incidence of antibiotic resistance and presence of virulence genes and biofilm formation. However, no relationship was observed between antibiotic resistance and biofilm formation. These data altogether suggest that occurrence of virulence gene did not seem to be necessary for the production of biofilm by GBS. Therefore, present study recommends routine monitoring of antimicrobial resistance and biofilm formation in order to understand the antibiotics suitable in the treatment of GBS infections.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable..

Availability of data and materials: All data generated or analysed during this study are included in this published article.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions

PY, VY, AKJ and SV conceived and designed the experiments. SV, and MK performed the experiments. Statistical analysis was carried out by AP and SV. PY and AKJ contributed to the interpretation of the results. PY and AKJ wrote and edit the manuscript in consultation with SV and VY.

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Figures

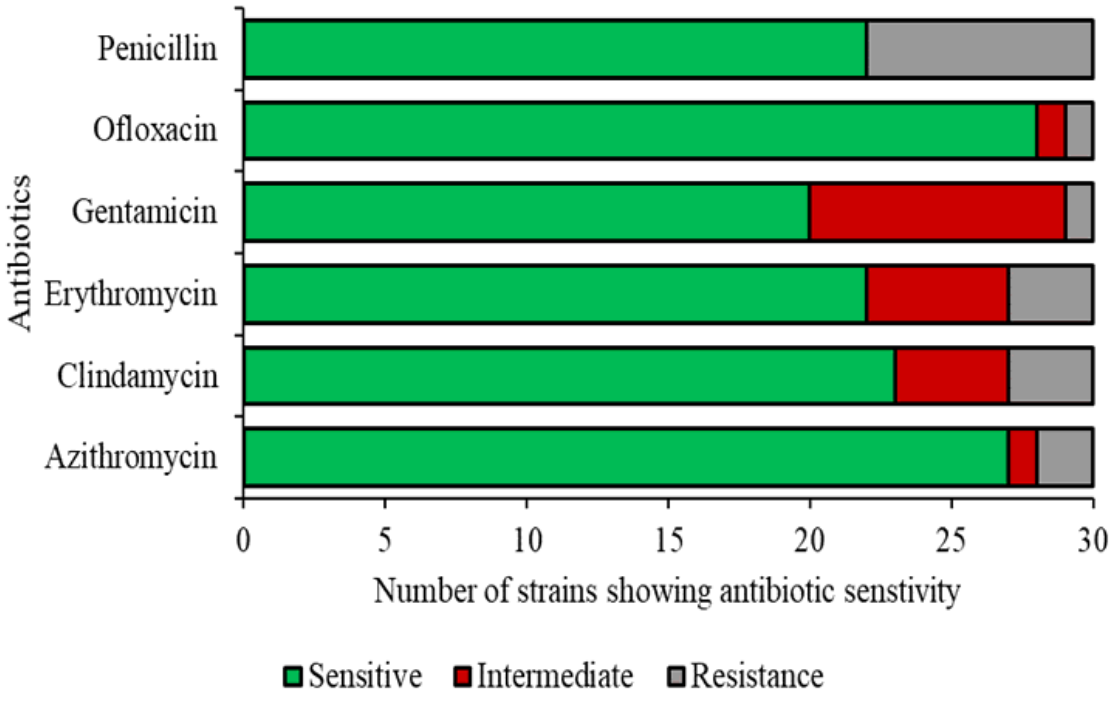

Figure 1

Figure 1

Antibiotic resistance phenotype of GBS isolates to different antibiotics examined in this study

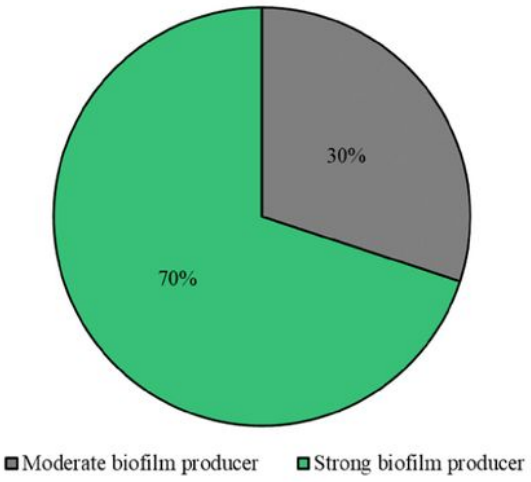

Figure 2a

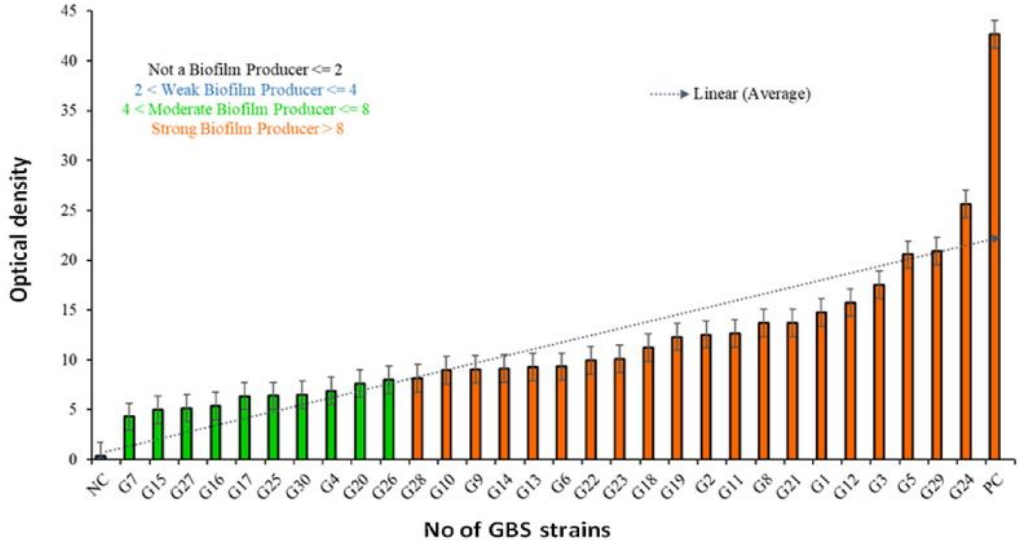

Figure 2b

Figure 2

a) Percentage of strong and moderate biofilm producer isolates b) Biofilm Crystal Violet Assay categorized the biofilm forming ability of GBS strains. Out of the 30 screened strains, G24 strain form strongest biofilm whereas G7 strain has the weakest biofilm forming ability. PC-positive control NC-negative control

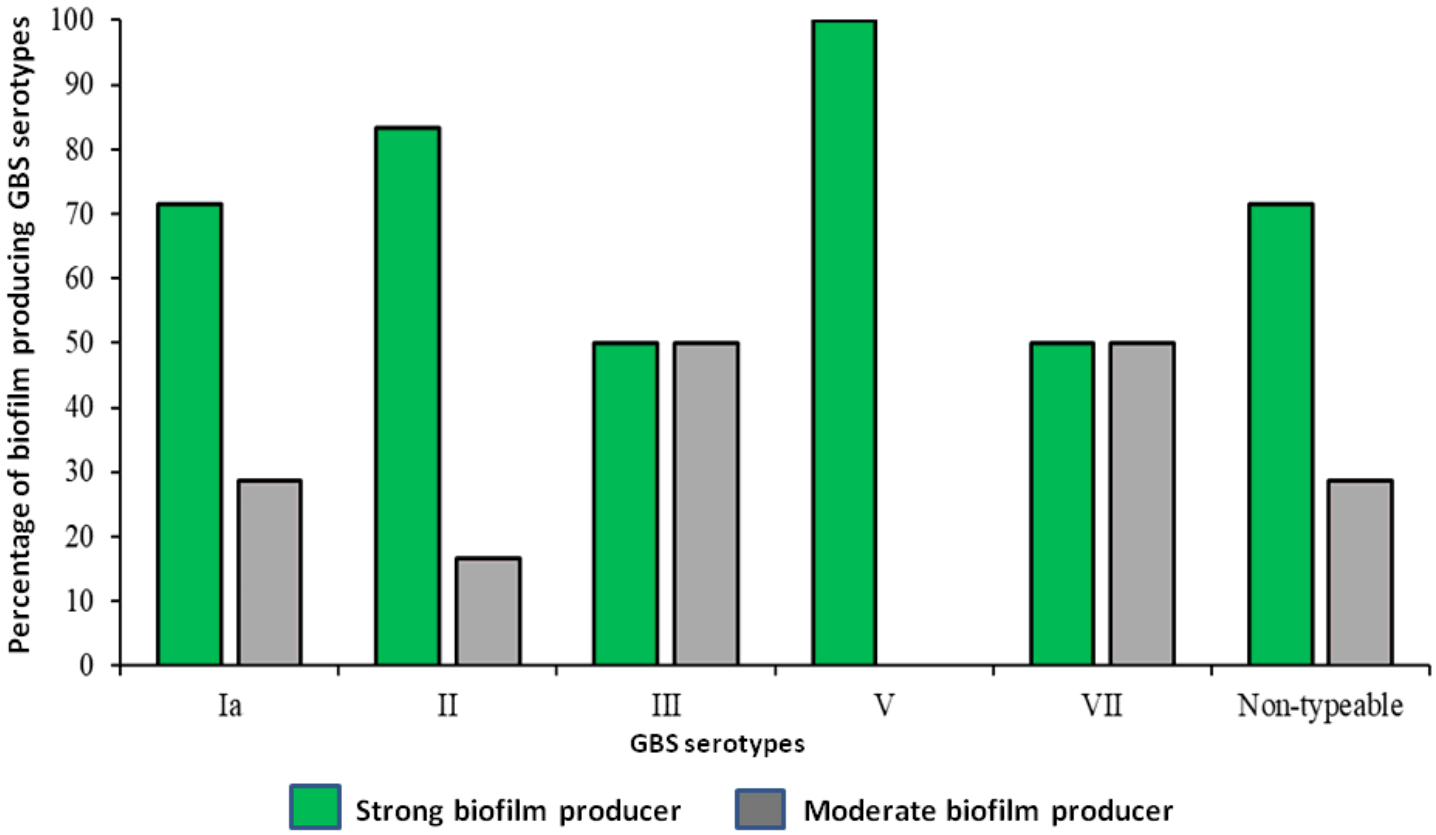

Figure 3

Figure 3

Biofilm producing phenotype of different GBS serotypes

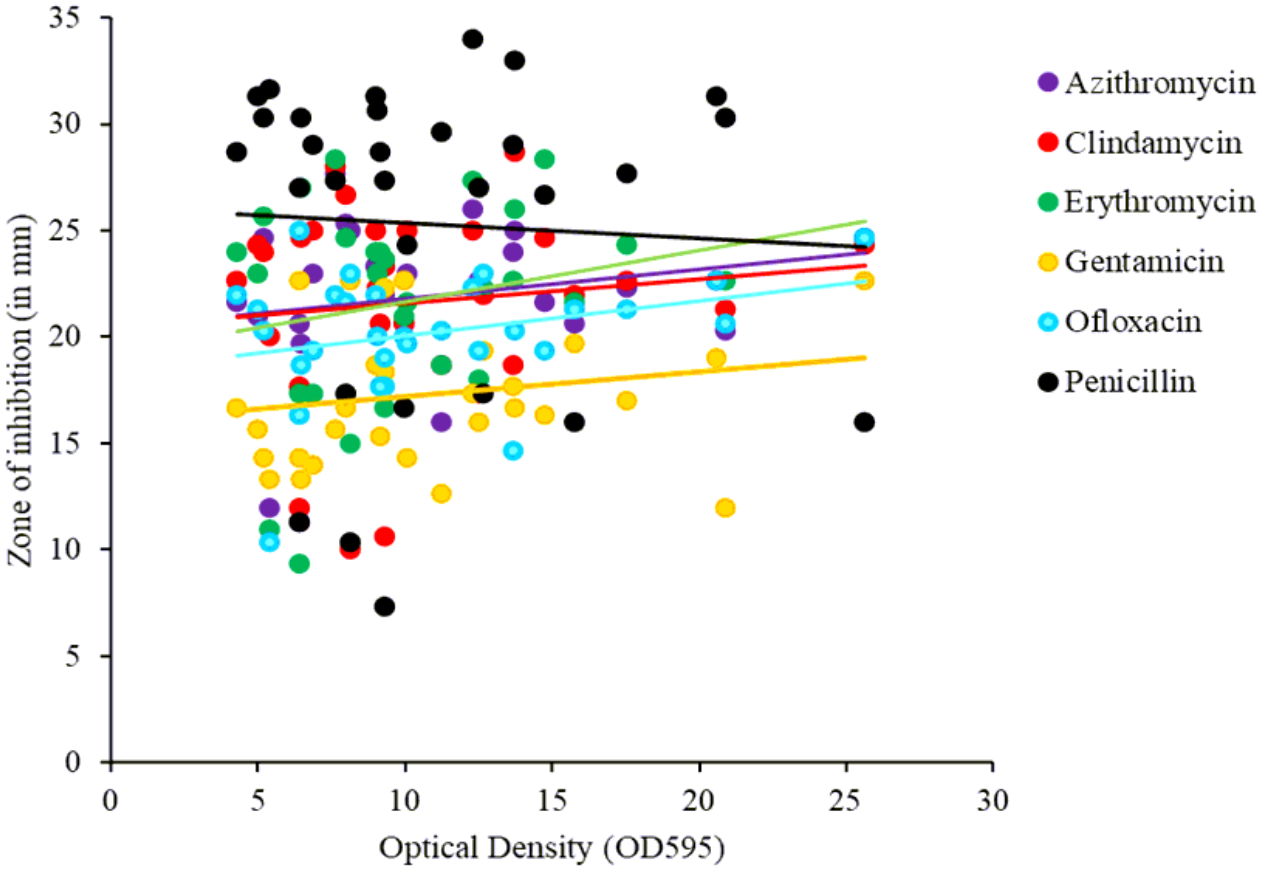

Figure 4

Figure 4

Statistical analysis to assess the correlation of antibiotic susceptibility with biofilm forming capacity in GBS isolates.

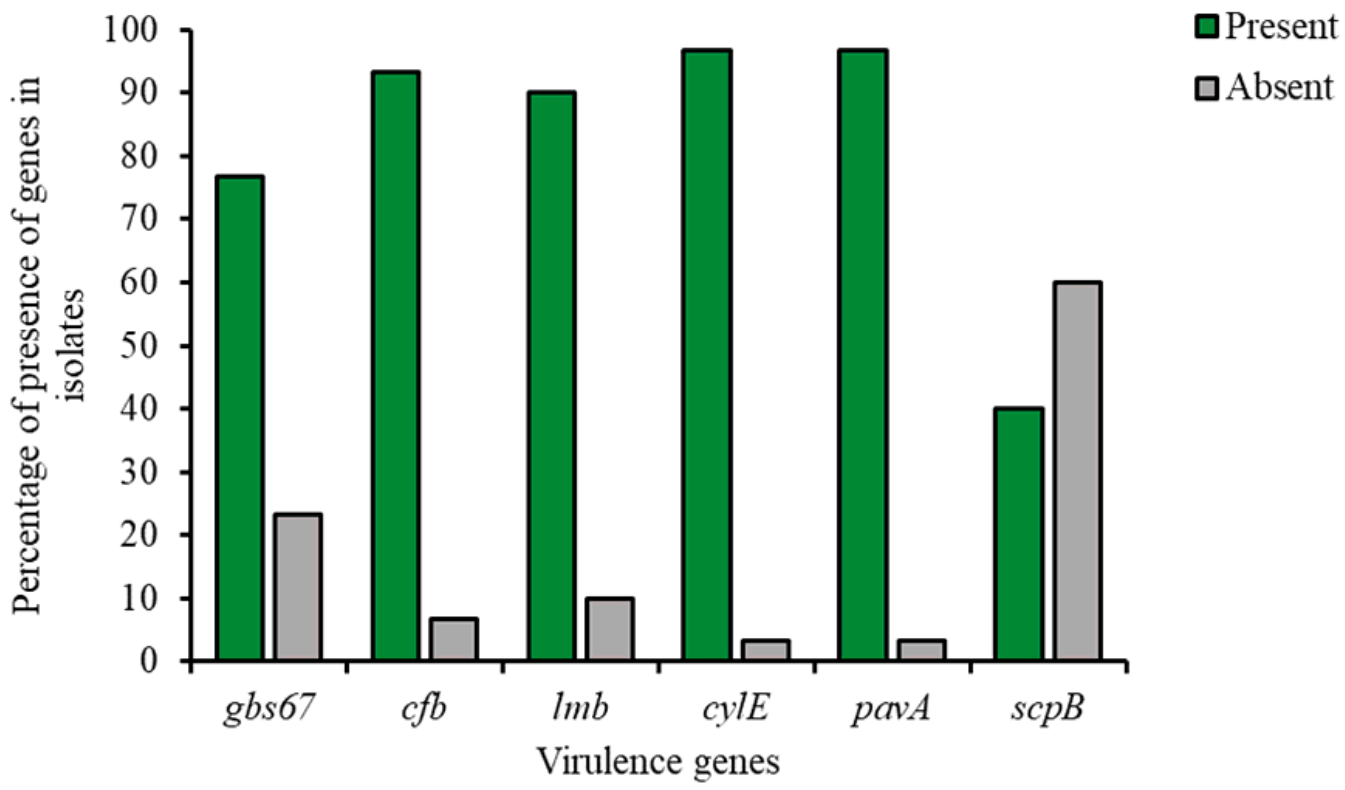

Figure 5

Figure 5

Distribution of virulence genes in all tested GBS isolates

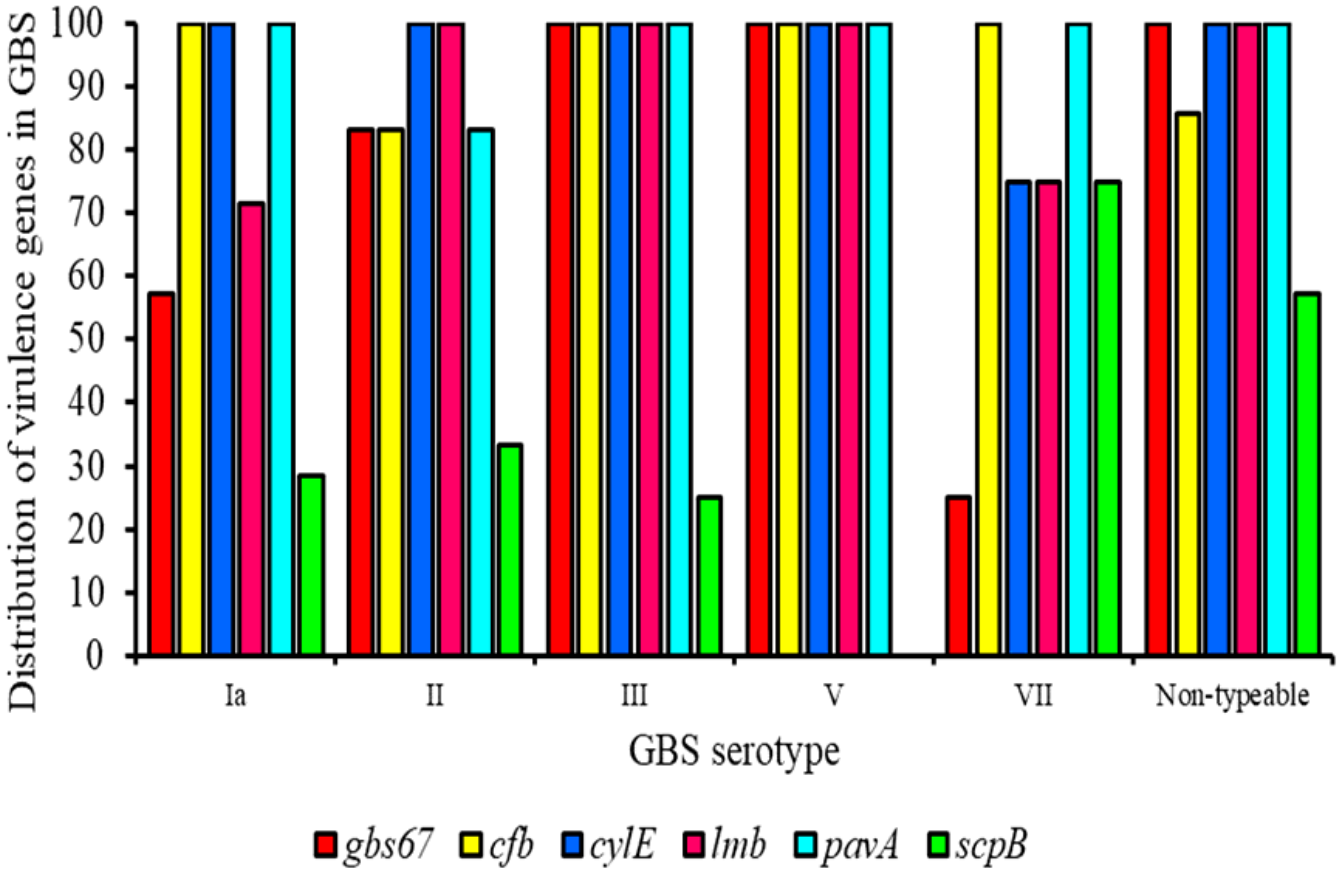

Figure 6

Figure 6

Occurrence of different virulence genes in GBS serotypes

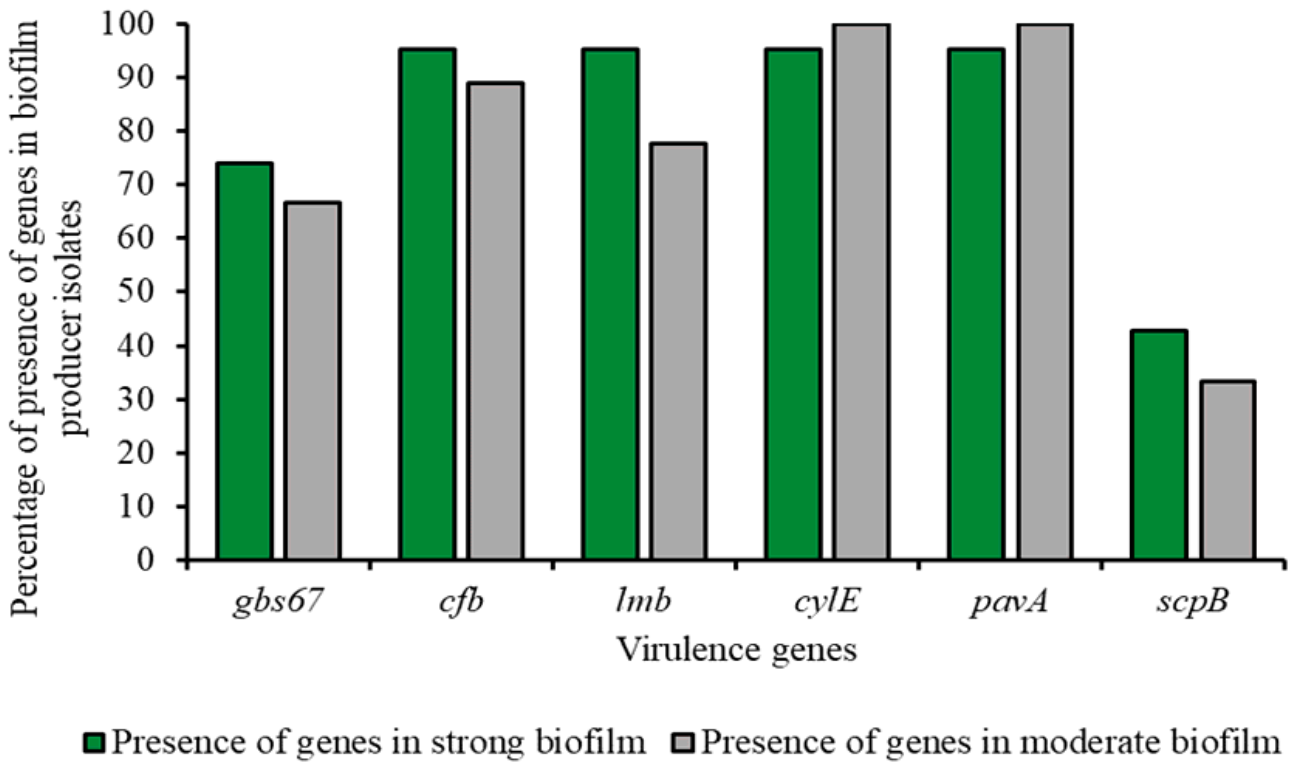

Figure 7

Figure 7

Distribution of virulence genes in strong and moderate biofilm producers GBS isolates.

Supplementary Files

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- Supplinfobmc1april2022.doc