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Isolation and Evaluation of Pilli Genes in Group B Streptococcus Isolated from Vaginal Samples

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ABSTRACT

Streptococcus agalactiae (Group B Streptococcus, GBS) continues to be an important colonizer and opportunistic pathogen in women with a growing focus on its potential virulence factors and changing antibiotic resistance profiles. Pili structures described by pilus island genes such as pi1 and pi2 are important factors for adhesion, colonization, and immune evasion and may be associated with trends of antimicrobial resistance. The study assessed the prevalence of pi1 and pi2 genes in S. agalactiae isolates retrieved from vaginal samples and its association with resistance against frequently used antibiotics in Iran. From the vaginal swabs of women attending gynecologic clinics at Al-Sadr Medical City, Najaf, Iraq, 60 S. agalactiae isolates were collected between summer 2024 and early winter 2025. Bacterial identification was carried out using standard cultural and biochemical methods. The Kirby–Bauer disc diffusion method was used to perform antibiotic susceptibility testing for penicillin G, ampicillin, erythromycin, clindamycin, and cefotaxime. Conventional PCR was used for molecular detection of pilus genes pi1 and pi2. Gene carriage and antimicrobial resistance associations were explored using statistical analyses. Penicillin G and ampicillin had low resistance rates (5.0 and 6.7%, respectively), while higher resistance rates were associated with erythromycin (23.3%), clindamycin (16.7%), and cefotaxime (11.7%). Overall, pi1 gene and pi2 gene were detected in 66.7% and 75.0% of isolates, respectively. There were significant associations for all pilus genes and both macrolides and lincosamides where pi2 was a stronger association ($p < 0.05$) across a wider range of antibiotics. Molecular surveillance is warranted due to the high prevalence of pilus genes among vaginal S. agalactiae isolates together with significant associations with antimicrobial resistance. These data identify pili as epidemiological markers and underscore the importance of regionalized antimicrobial stewardship to minimize the spread of resistant GBS lineages.

INTRODUCTION

Group B Streptococcus (GBS), or Streptococcus agalactiae, is a Gram-positive bacterium that asymptomatically colonizes the genitourinary and gastrointestinal tracts of 10-30% of healthy adults, particularly women (Vazquez-Guillen et al., 2024). Although a commensal organism, GBS remains an important pathogen in perinatal medicine due to maternal vaginal–rectal colonization being the natural reservoir for vertical transmission during childbirth leading to early-onset sepsis, pneumonia, and meningitis (Verma et al., 2023). The acquisition of colonization of the maternal vaginal tract is therefore a pivotal step in the pathogenesis of GBS disease in the neonate and is a target of interventions such as antibiotic prophylaxis and, hopefully in the near future, vaccination (Patras & Nizet, 2018).

GBS is naturally present in the vaginal niche and one of the major determinants of its virulence are surface structures, including pili (also known as fimbriae) which have been implicated in the vaginal colonisation process. Pili in GBS pili are characterized as

filamentous protein appendages that promote adhesion to host epithelial cells, biofilm formation, invasion and evasion of the immune response. GBS pili are comprised of three types of subunits: a tip adhesin (PilA), a backbone protein (PilB), and a basal, or anchoring protein (PilC), that are covalently assembled through the action of class C sortases (Khodaei et al., 2008; Goh et al., 2024). For example, these pilus-encoding genes are clustered in genomic areas termed "pilus islands" (PIs) with predominance of PI-1 and PI-2 variants (PI-2a or PI-2b) that are variably distributed among strains (Rosini & Margarit, 2018).

A great body of recent molecular epidemiology and virulence-factor research has shown that the presence of pilus islands is closely associated with strain invasiveness, serotype and colonization potential. For example, a massive study performed in Iran showed that PI-1 + PI-2a and PI-1 + PI-2b combinations were prevalent in colonising and invasive GBS isolates, suggesting that the majority of clinical strains contain one or two pilus islands (Nabavinia et al., 2020). Likewise, in South Korea invasive strains (notably serotype III/ST-17) were positively associated with PI-2b with evidence for a serotype specific virulence signature (Kim et al., 2024). Together, these data indicate that pili are more than a simple means of surface adherence and actually organize GBS populations in terms of pathogenicity, which has potential implications for diagnostic and vaccine development.

In addition to having a role in static adhesion, pili are also involved in biofilm formation—a multicellular behavior that increases bacterial survival in unfavorable environments such as the vaginal mucosal surface. Biofilms are associated with a virulence mechanism of GBS to evade the action of the host and survive in time. Indeed, weak biofilm producers emerged from hypervirulent lineages (e.g., ST-17) while strong biofilm-forming strains were more frequently recovered from asymptomatic carriers, thus supporting the idea of a trade-off between colonization and invasiveness (Motallebirad et al., 2021).

Functional studies also emphasize the biological significance of pilus genes. The isogenic GBS mutants in question included ΔpilA or ΔpilC (lacking pilus subunits), and experimental work using these mutants showed significant loss of adherence to epithelial cells and vaginal persistence in in vivo mouse models (other work original but cited later; (Patras & Nizet, 2018). These results were augmented by studies of the accessory role of serine-rich repeat (Srr) proteins, glycosylated adhesins that also promote colonization. When tested for competition in the animal model, mutants deficient in pilus or Srr proteins were rapidly outcompeted by wild-type strains, thus furthering the hypothesis that pili and Srr adhesins were synergistic for the establishment of vaginal colonization (Sheen et al., 2011).

Most recently, high-throughput sequencing of the genomes of multiple GBS strains has led to the identification of new pilus associated gene clusters that enhance the traditional model of GBS virulence. Following a transposon-mutagenesis screen in the A909 strain, a pilus cluster that also included a backbone pilin gene (BP), an assembly accessory protein gene (AP1), and sortase gene (SrtC), was demonstrated to significantly modulate adhesion and virulence in a murine model. Such discoveries highlight the genetic plasticity of GBS while adding surprising levels of complexity to pilus biology in this organism as they reveal pilus module diversity beyond the canonicial PI-1 and PI-2 loci that may regulate colonization and disease (Xu et al. 2025).

The current study was performed to detect pilli genes (pi1 and pi2) in the isolates of *Streptococcus agalactiae* taken from vaginal samples of women in the City of Najaf in Iraq. It is also an attempt to estimate a correlation between these genes and specific antimicrobial resistance profiles.

PATIENTS AND METHODS

Study Design and Setting

A cross-sectional study was conducted in the period from the November of 2024 until the September of 2025 at Al-Sadr Medical City Hospital, Najaf Governorate, Iraq. Sixty women who presented for routine examination at gynecology and obstetrics clinics or vaginal discomfort were recruited. Sample: Inclusion Criteria: 18–45-year-old. Patients were excluded if they had antibiotic use in the previous week, pregnancy-related complications, chronic illness (eg, diabetes or autoimmune disease), or concomitant bacterial or fungal infections.

A 75-ml sterile cotton swab was used to collect vaginal swab samples aseptically and then immediately placed in Amies transport medium. Microbiology Laboratory of Al-Sadr Medical City processed all samples. Specimen swabs were inoculated onto Columbia Blood Agar and Granada Medium (Oxoid, UK), the latter being selective for Streptococcus agalactiae, and cultured aerobically at 35–37 °C for 18–24 hours with 5% CO_2.

Presumptive GBS colonies (orange-red on Granada medium, β-hemolytic on blood agar, Gram positive cocci in chain) were subjected to further tests for confirmation like CAMP test, catalase reaction, bacitracin resistance, and latex agglutination serogrouping (Remel™ Streptococcal Grouping Kit). Confirmed isolates were stored as tryptic soy broth with 20% glycerol at −80 °C for further molecular studies. The quality-control strain was Streptococcus agalactiae ATCC® 13813™.

For GBS isolates, the antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar enriched with 5% sheep blood, in accordance with CLSI (2024). We tested for the antibiotics listed below, commonly used for the treatment of GBS infections:

Penicillin G (10 U) – Oxoid, UK

Ampicillin (10 µg) − Turkey, Bioanalyse

Erythromycin (15 µg) – Mast Diagnostics, UK.

Clindamycin (2 µg) — Bio-Rad, France.
Cefotaxime (30 µg)– HiMedia, India
After the incubation period of 20–24 hours, zone diameters were recorded and the isolates were categorized as susceptible, intermediate, or resistant. The D-test was used to assess for inducible clindamycin resistance. S. pneumoniae (ATCC® 49619™) was used as a control reference strain for susceptibility testing.
Genomic DNA of all confirmed GBS isolates were extracted using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania) according to manufacturer instructions. The DNA concentration and purity were confirmed by a Nanodrop spectrophotometer (ThermoScientific, USA), and the sample stored at −20 °C until subjected to PCR detection.
Detection of the pilus islands pi1 and pi2 was performed by conventional PCR assays. Primers which target the backbone pilin genes from the PI-1 and PI-2 operons were selected from previously published protocols. All PCR reactions were in a volume of 25 µL that included: 2× PCR Master Mix, Promega, USA: 12.5 µL)
1 µL of each forward and reverse primer (10 pmol/µL), 3 µL template of extracted DNA, and other reagents for a total reaction volume of 50 µl.
Cycling conditions:(1) PCR amplification was performed using an Eppendorf Mastercycler (Germany) as follows: Step 1 — Initial denaturation 95 °C 5 min;
35 cycles of denaturation (94 °C, 30 seconds), annealing (56 °C, 40 seconds), and extension (72 °C, 45 seconds); Final extension at 72 °C for 7 min.
The PCR products were separated on 1.5% agarose gel containing ethidium bromide and observed with a UV transilluminator (Bio-Rad GelDoc System). Positivity was determined by the presence of particular band sizes representing pi1 and pi2. Streptococcus agalactiae ATCC® 13813™ (pi1/pi2-positive) was used as a positive control and nuclease-free water was included as a negative control (table 1).

Ethical Considerations

Ethical approval was obtained from the Institutional Review Board of Al-Sadr Medical City, and written informed consent was obtained from all participants prior to sample collection. Confidentiality and anonymity were maintained throughout the study.

Table1: List of primers applied for amplification of pilli genes

Gene	Amplicon size (bp)	Annealing temp (°C)	Primer sequence (5′–3′)
pi1	**~520 bp**	**58°C**	F: *AGTGCTGCTGATGTTGTTGA* R: *CCTTCTTGCTTTGCTGATGT*
pi2	**~680 bp**	**57°C**	F: *TTGGAAGAGGTTGCTGAAGA* R: *ACCATCTTGCTTTCCTGTCA*

RESULTS

The study showed that 5% of isolates were penicillin G resistant, while 95% were sensitive to penicillin G. For ampicillin, a similar trend was observed with 6.7% of the isolates found to be resistant and 93.3% susceptible. The highest resistance (23.3%) and susceptibility (76.7%) were recorded for Erythromycin. Of these, 16.7% were resistant to clindamycin, 83.3% of isolates were susceptible to it. Resistance to cefotaxime was present in 10% of all isolates, and 90% of the isolates were susceptible to cefotaxime (Table 2).

Table 2. Percentage of antibiotic resistance recorded in the isolates of *Streptococcus agalactiae*

Groups	Resistant Isolates No. (%)	Susceptible Isolates No. (%)
Penicillin G	**3 (5%)**	**57 (95%)**
Ampicillin	**4 (6.7%)**	**56 (93.3%)**
Erythromycin	**14 (23.3%)**	**46 (76.7%)**
Clindamycin	**10 (16.7%)**	**50 (83.3%)**
Cefotaxime	**6 (10%)**	**54 (90%)**

Among the examined Streptococcus agalactiae isolates, the pilus island gene pi1 was identified in 66.7% (40/60) of samples, whereas 33.3% (20/60) tested negative for this gene. In comparison, the pi2 gene showed a higher detection frequency, being present in 75.0% (45/60) of isolates and absent in 25.0% (15/60). Overall, the distribution demonstrates that both pilus genes are commonly found within the isolate set, with pi2 showing a relatively greater proportion of positive isolates. (Table 3).

Table 3. Detection of pilli genes in the Streptococcus agalactiae isolates

Groups	Positive No. (%)	Negative No. (%)
pi1	40 (66.7%)	20 (33.3%)
pi2	45 (75.0%)	15 (25.0%)

Table 4 presents the association analysis shows variable chi-square values for the linkage between antibiotic resistance patterns and the presence of pili genes among *Streptococcus agalactiae* isolates. For the pi1 gene, significant associations were observed with Penicillin G ($p = 0.042$), Erythromycin ($p = 0.002$), and Clindamycin ($p = 0.011$), while Ampicillin and Cefotaxime demonstrated non-significant associations. In contrast, the pi2 gene exhibited broader significance, with Penicillin G ($p = 0.021$), Ampicillin ($p = 0.009$), Erythromycin ($p < 0.001$), Clindamycin ($p = 0.001$), and Cefotaxime ($p = 0.026$) all showing statistically significant associations. Overall, a higher number of significant associations were recorded for pi2 compared to pi1, reflecting differing distribution patterns of antibiotic resistance across isolates carrying each gene.

Table 4. Association between antibiotic resistance and Presence of pili genes in the isolates of *Streptococcus agalactiae*

Groups	pi1 Chi Square (P value)	Pi2 Chi Square (P value)
Penicillin G	4.12 (0.042)*	5.36 (0.021)*
Ampicillin	3.45 (0.063)	6.82 (0.009)*
Erythromycin	9.74 (0.002)**	12.55 (<0.001)**
Clindamycin	7.18 (0.011)*	10.46 (0.001)**
Cefotaxime	2.84 (0.092)	4.95 (0.026)*

*** Significant at P<0.05 ; ** High significant at P<0.001**

DISCUSSION

In the current study, we showed that pilus genes were identified in a considerable fraction of S. agalactiae vaginal isolates (66.7% for pi1 and 75.0% for pi2), and we specifically found low rates of β-lactam resistance (5.0% for penicillin G; 6.7% for ampicillin) and relatively high rates of resistance to macrolides and lincosamides (23.3% for erythromycin; 16.7% for clindamycin). In particular, 6 pilus genes exhibiting pilus type-specific associations were identified, with pi2 having stronger and more numerous statistically significant associations with resistance phenotypes compared to pi1. There was a moderate positive association between pilus carriage and resistance to erythromycin and clindamycin (Table 4). The present results are in line with and build on recent findings on the epidemiology, virulence genetics and antimicrobial susceptibility of GBS from different geographical locations.

The high prevalence of pilus island in our cohort (frequent identification of both pi1 and pi2) is in agreement with molecular epidemiology studies demonstrating that most clinical GBS strains carries at least one pilus island and that both PI-1 and PI-2 types are frequently identified among colonising isolates (Shabayek & Spellerberg, 2018; Nabavinia et al., 2020). For instance, Shabayek & Spellerberg summarized that pilus islands are broadly distributed throughout GBS lineages and play roles in colonization, biofilm formation, and immune evasion (Shabayek & Spellerberg, 2018). In a number of regional studies PI-1 + PI-2a form the majority of vaginal isolates, whereas the distributions of PI-2b or PI-2a differ by clone and geography (Nabavinia et al., 2020; Panahi et al., 2023). In line with such regional variation, our detection rates (pi2 slightly more prevalent than pi1) maybe reflect that the pilus repertoire in this Iraqi population is akin to that seen in some other Middle Eastern and Asian cohorts.

The antimicrobial susceptibility patterns observed in this study (i.e., near-universal susceptibility to β-lactams, variable susceptibility to erythromycin and clindamycin) are also consistent with contemporary literature. Reported rates of penicillin and ampicillin remain excellent nationally and regionally (Petca et al., 2024; Motallebirad et al., 2021), though reported rates of macrolide and lincosamide (ML) resistance have increased and show significant geographic variability. The approximately 23% erythromycin resistance rate and 17% clindamycin resistance seen in our isolates are in ranges observed within several recent investigations, which have reported macrolide resistance often between ~10–30% depending on region and time frame (Nabavinia et al., 2020; Panahi et al., 2023). These results highlight the remaining utility of β-lactams as first-line agents and the importance of testing for macrolide/lincosamide susceptibility when these agents have been considered as potential alternatives for the treatment of β-lactam allergic patients.

Proven associations between pilus genes, especially pi2, and antibiotic resistance are mentioned. There are also multiple recent studies that have associated pilus island profiles with other genetic determinants, such as antimicrobial resistance genes or lineage-associated resistance phenotypes (Panahi et al., 2023;0 Nabavinia et al., 2020). Consistent with the idea that some clonal lineages combine specific pilus genotypes with resistance determinants, likely due to linkage on mobile elements or clonal expansion of successful genotypes (Shabayek & Spellerberg, 2018), we found stronger chi-square associations for pi2 across multiple antibiotics

(including erythromycin, clindamycin, and, to a lesser extent, cefotaxime). However, cross-sectional association does not establish a causal link, and molecular typing (e.g., MLST, whole-genome sequencing) would be necessary to determine whether pilus loci and resistance markers co-locate in this population on the same genetic background or mobile elements.

Other authors have also concluded that some resistance patterns are better reflected in the pilus–serotype comparison of the prevalent (colonic) strains compared to the invasive clones, for example ST-17 appears to carry different pilii–serotype combinations, and occasionally different resitance profiles (Motallebirad et al., 2021). In accordance with the epidemic similarities we defined, studies conducted in Iran and neighboring areas have indicated higher levels of erythromycin and clindamycin resistance with greater prevalence of pilus islands (Nabavinia et al., 2020; Panahi et al., 2023). In a wider context, meta-analyses and reviews highlight substantial heterogeneity between countries in resistance trends and advocate for availability of local surveillance data to guide empiric choices and prophylaxis polices.

This study has limitations that deserve mention. Generalizability is limited both by the cross-sectional design and the sample size (n = 60), with implications for temporal inference and precision of association estimates. However, phenotypic resistance described only through disc diffusion; and molecular detection of erm and mef, or mucosal inducible resistance and/or genomic lineage assignment were not performed in this study and would improve causal inference regarding gene–resistance linkage. Detection of pilin genes by PCR confirms only presence, not expression; functional assays (adhesion, biofilm, or animal models) would confirm their phenotypic relevance to colonization and persistence.

CONCLUSION

The current study—isolation and characterization of pilus genes in GBS from vaginal isolates—fills a significant knowledge gap in maternal–neonatal infection research, integrating molecular epidemiology, virulence genetics, and possible translational relevance. Understanding local GBS persistence, pathogenesis, and the foundation for future strain-specific preventive strategies would be beneficial by determining which combinations of pilus islands/variant subunit gene and vaginal colonising strains predominate.

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