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## ***Streptococcus agalactiae* pulsed-field gel electrophoresis patterns cross capsular types**

P. PILLAI<sup>1</sup>, U. SRINIVASAN<sup>1</sup>, L. ZHANG<sup>1</sup>, S.M. BORCHARDT<sup>2</sup>, J. DEBUSSCHER<sup>1</sup>, C. F. MARRS<sup>1</sup>, and B. FOXMAN<sup>1,\*</sup>

<sup>1</sup>Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, MI, USA

<sup>2</sup>Fargo VA Medical Center, Fargo, ND, USA

### **SUMMARY**

*Streptococcus agalactiae* is a genetically diverse organism; when typed by pulsed-field gel electrophoresis (PFGE), multiple types appear within a single serotype. We tested whether *S. agalactiae* PFGE types correspond to a specific serotype within individuals, and different individuals from the same geographic area. A total of 872 *S. agalactiae* isolates from 152 healthy individuals were classified by PFGE and capsular serotype. Serotype V was the most homogeneous (Simpson's diversity index 0.54); and types III, II and Ib were mostly heterogeneous (Simpson's diversity index 0.90). Within an individual, isolates with the same PFGE patterns had identical capsular types, but across individuals the same PFGE types sometimes occurred in different serotypes. Capsular type alone is insufficient to define epidemiological relatedness. Although PFGE types appear to be a valid surrogate for capsular typing of isolates from the same individual, it is not a valid surrogate for serotype in isolates from different individuals.

### **Keywords**

Diversity; Group B *Streptococcus*

### **INTRODUCTION**

*Streptococcus agalactiae*, also called Group B *Streptococcus* (GBS), is a facultative Gram-positive coccus distinguished from other types of *Streptococcus* by the presence of a type B-specific carbohydrate antigen known as the C substance [1, 2]. There are nine known serotypes, the distribution of which vary with geographic location, although serotypes Ia, II, III and V predominate in invasive disease [3]. *S. agalactiae* is a common colonist of the gastrointestinal and genitourinary tracts, and can be found in up to 40% of the female population and 30% of the male population [4, 5]. It was first noted in the 1970s as a leading cause of neonatal disease but over the last two decades the species has emerged as an invasive pathogen in pregnant women and the elderly [6, 7]. While the incidence of early-onset neonatal disease and infection in pregnant women has declined over the past decade

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\* Author for correspondence: Professor B. Foxman, SPH 1 Tower, Rm 5607, 109 Observatory St, Ann Arbor, MI 48109, USA. (bfoxman@umich.edu).

### **DECLARATION OF INTEREST**

None.

due to prenatal screening, that of late-onset neonatal disease has remained stable, and the incidence of disease in the elderly has increased [6, 8–12].

*S. agalactiae* is a genetically diverse organism. When characterized using pulsed-field gel electrophoresis (PFGE) multiple PFGE types often share the same serotype [13, 14]. However, the level of genetic diversity within the various molecular capsular types, as characterized by differing PFGE types, remains unclear. Previous studies have used PFGE types as a surrogate for serotyping, presuming that isolates from a single individual with the same PFGE type have the same serotype [5, 15]; however, this presumption has been based on the examination of a small number of isolates [16]. Given the importance of correctly characterizing capsular types, both for understanding transmission dynamics and vaccine formulation, there is a need to validate the presumption that isolates from one individual with the same PFGE type have the same capsular type.

Over time, the proportion of *S. agalactiae* isolates that are non-typable using the Lancefield serotyping method has increased [10], suggesting that the use of PFGE to infer capsular type in different individuals may be desirable. However, it is noteworthy that one study reported a single mother–child pair of isolates that were identical by PFGE but with different serotypes [13]. The capsular serotype of a strain could potentially be altered by as little as the horizontal transfer of a single gene [17, 18] which might not result in a change in PFGE type. Therefore the reliability of using PFGE profiles as a predictor of capsular type for isolates from different individuals, even from within the same geographic area, warrants investigation.

We examined a large collection of *S. agalactiae* isolates from individual cases in order to describe the genetic diversity of these isolates and test the hypotheses that *S. agalactiae* PFGE types correspond to a specific serotype within an individual, and between different individuals from the same geographic area.

## METHODS

### Study protocol

As described previously [19, 20], 738 male and female University of Michigan students were recruited to provide specimens from throat and mouth, vagina, anal orifice, and urine samples. In total, 913 *S. agalactiae* isolates were collected over a 12-week period, a subset of which was from students who provided multiple samples over the study period. Isolates were serologically confirmed with the Slidex Strepto B kit (bioMérieux, USA).

### PFGE

Of the 913 original isolates, 882 were typed using PFGE and patterns were analysed for relatedness by visual inspection and using the BioNumerics software (Applied Maths, Belgium) as described previously [5, 20]. A subset of the isolates either failed to yield restriction fragments with *Sma*I (13), or were not analysed with BioNumerics (6), or were not viable at the time of the present study (12).

### Capsular typing using library on a slide hybridization

Sixteen of the isolates from the original collection were not viable. The remaining 897 isolates were grown in 9 ml Todd–Hewitt broth (THB) (BD, USA) at 37 °C in 5% CO<sub>2</sub> for 36 h. Genomic DNA was extracted and purified using sonication and heat treatment as previously described [21]. The DNA from each isolate was arrayed in duplicate on Vivid Gene Array slides with nylon membranes (Pall, USA) using the VersArray ChipWriter Compact system (Bio- Rad, USA).

PCR amplification of probe sequences was performed as follows: DNA lysates were prepared from 3 ml *S. agalactiae* overnight culture in THB. Each reaction mixture (48  $\mu$ l) included 1  $\mu$ l of each primer (25 pmol), 1  $\mu$ l DNA lysate, and 45  $\mu$ l Platinum PCR SuperMix (Invitrogen, USA). The primers and PCR conditions used to amplify capsular types Ia, Ib, II, III, IV, V and VI–VIII genes were as described previously [12, 22], with the following modifications: 94 °C for 30 s, 49 °C (type V) or 55 °C (type III) for 30 s, and 72 °C for 25 s, for 35 cycles. PCR product (10  $\mu$ l) was analysed by electrophoresis on a 1% agarose gel, stained with 0.02  $\mu$ g ethidium bromide/ml, and visualized by UV transillumination.

PCR amplification products were purified using the QIAquick gel extraction kit and the QIAquick PCR purification kit (Qiagen Inc., USA) and fluorescein labelled with 2.5  $\mu$ l of 1mM fluorescein-12-dCTP (PerkinElmer, USA) using a modification of the BioPrime DNA Labeling System (Invitrogen).

The probes were hybridized to genomic DNA arrayed on slides in duplicate using previously described methods [23] and hybridization signals were detected using a ArrayIt Microarray SpotWare Colorimetric Scanner (TeleChem International Inc., USA) and analysed with IconoClust software (Clondiag Chip Technologies, Germany). The signal intensity of the spots was adjusted for background and normalized to the signal intensity of a quantification probe which consisted of a mixture of *S. agalactiae* housekeeping genes (*adhP*, *glcK*, *glnA*, *pheS*). These values were used to account for differences in genomic DNA concentrations at different spots. Cut-offs for probe positive strains were established using previously published methods [24].

A hierarchical strategy for capsular-type confirmation was employed, such that at least one isolate with a unique PFGE pattern was assigned a capsular type via traditional dot-blot hybridization, in addition to the Library on a Slide dot-blot hybridization. Isolates with non-confirmatory capsular-type results in the Library on a Slide assay or that were discrepant with both traditional and Library on a Slide were confirmed by PCR and dot-blot hybridization methods using the primer sequences and methods described earlier [12, 22]; 892/897 isolates originally printed on the Library on a Slide were confirmed using the hierarchical typing strategy and were assigned a unique capsular-type designation.

## RESULTS

Of the 892 isolates assigned a unique capsular type and 882 isolates assigned a PFGE pattern, a total of 872 *S. agalactiae* isolates from 152 individuals had both PFGE pattern data and a unique capsular-type assignment. These 872 isolates were used for the final analyses. Overall, an average of six isolates were available from each individual.

### Capsular type

The capsular-type distribution of the *S. agalactiae* isolates was examined in the final collection. In order to account for the possibility that an individual was persistently colonized with the same organism over the course of the study, the capsular-type distribution of the collection was also examined by choosing one isolate from each unique PFGE pattern from a given individual (168). The capsular-type distributions were very similar whether analysing one isolate or all isolates per person, suggesting few individuals were colonized with more than one serotype (Fig. 1).

As reported previously, based on results of traditional dot-blot hybridization to determine capsular type [20], there was a predominance of type V isolates (322, 36.9%). Additionally the collection included type Ia (170, 19.4%), type III (124, 14.2%), type Ib (123, 14.1%),

type II (88, 10.1%), type VIII (10, 1.1%) and type IV (8, 0.9%). Only 27 isolates from the entire collection were non-typable (3%) by all molecular capsular-typing strategies. Interestingly, while a majority of the isolates from a given individual had the same capsular type, nine (5.9%) of the 152 individuals were concurrently colonized with two different capsular types; an additional two individuals were colonized with two different capsular types over the course of the study.

### Diversity within capsular type

Fifty-two PFGE patterns were identified in the 872 isolates. To explore genetic diversity we chose one representative isolate for each PFGE pattern from each individual, resulting in a total of 168 isolates with both a PFGE type and a capsular type. Based on Simpson's diversity index, which indicates the probability of randomly choosing two isolates of a given capsular type with a different PFGE type [25, 26], capsular types III, II and Ib exhibited the greatest heterogeneity; while capsular type V isolates were the most homogeneous (Table 1).

### PFGE type within individuals

Fourteen of the 152 individuals (9.2%) carried isolates with more than one PFGE type. In three of these individuals, all isolates, which included an average of seven isolates per person, were of the same capsular type. In the remaining 11 individuals, one had three isolates with unique PFGE patterns, representing two distinct capsular types, nine persons had two PFGE types representing two distinct capsular types (Fig. 2), and one individual had 11 isolates representing two PFGE types and two serotypes. Two of the isolates sharing PFGE types had different serotypes. There was a 2.0% probability of an individual having more than one unique PFGE type but representing a single capsular type and a 6.6% probability of an individual having more than one unique PFGE type each representing a distinct capsular type. We observed one individual (1/152) where both these phenomena occurred (0.7%). Therefore, within a given individual, while differing PFGE patterns potentially represented identical capsular types, the probability that all isolates with a unique PFGE pattern represent the same capsular type was 99%, which may reflect technical rather than true biological variability.

### PFGE type in individuals

When examining the distribution of capsular types and PFGE patterns across individuals, 10/52 (19.2%) identified PFGE patterns occurred in more than one capsular type. Seven PFGE patterns included two capsular types, while three PFGE patterns included three capsular types. Based on these results, there could be between an 8.3% and 15.4% error rate in capsular-type assignment if PFGE was used as a surrogate for capsular typing in these individuals from similar geographic areas.

## DISCUSSION

DNA sequencing of various strains of *S. agalactiae* has demonstrated the genetic diversity of the species and isolates of different serotypes have been noted to share more sequence similarities than isolates of the same serotype [27, 28]. Tettelin *et al.* suggest that the level of *S. agalactiae* genetic diversity and evolutionary patterns are not adequately reflected with serotype classification [27]; thus the level of heterogeneity within serotype classification could vary widely. We describe the heterogeneity of *S. agalactiae* and the impact of this heterogeneity on scientific inference by examining the PFGE patterns and capsular types of 872 isolates from 152 individuals. *S. agalactiae* is highly heterogeneous but the heterogeneity varies by capsular type, with the greatest heterogeneity occurring in capsular types III, Ib and II. Within an individual, isolates with the same PFGE patterns had identical capsular types, while across individuals PFGE types appeared in more than one capsular

type. Therefore, capsular type alone was not sufficient to characterize epidemiological relatedness. Although PFGE types appear to be a valid surrogate for capsular typing of isolates from the same individual, it is not a valid surrogate for serotype in isolates from different individuals.

The heterogeneity we observed is consistent with previous reports describing much smaller collections [14, 29], and as observed in these earlier studies, capsular type V was the most homogeneous [14, 30]. These studies did not report a diversity index score and so we cannot make a direct comparison with our study findings. Interestingly, the relative homogeneity of type V isolates is consistent with the emergence of a predominant capsular type V clonal lineage [31].

Assuming that a common PFGE type represented the same capsular type across individuals would have resulted in an error rate in capsular-type assignment of as much as 15%. Thus, using 2–3 band differences in PFGE pattern might not be sufficiently stringent for outbreak investigations [32], as geographically and temporally linked isolates with distinct serotypes shared almost identical PFGE patterns, providing a strong potential for linking epidemiologically unrelated strains and obscuring the role and routes of transmission in colonization and disease.

Multiple researchers have demonstrated that *S. agalactiae* isolates sharing identical multilocus sequence types (MLST) can possibly represent differing capsular types [18, 33, 34]. Jones *et al.* described the increased potential of *S. agalactiae* sequence type 17 to cause invasive disease within neonates when compared to other sequence types independent of capsular type [35]. Given that *S. agalactiae* capsule is a key determinant of virulence and vaccine development is based on capsular type, it is important to recognize that MLST typing is likely to encounter similar problems as PFGE typing if used as a surrogate for capsular typing.

Our study collection was large, enabling us to characterize the genetic diversity more precisely than had been done previously. Furthermore, we quantified the degree of diversity using Simpson's diversity index. The consistent effect across a large number of isolates strongly supports the hypothesis that PFGE typing distinguishes between capsular types isolated from a single individual. Using the hierarchical capsular-typing strategy we employed of dot-blot hybridization and PCR, only 27 isolates (3%) of our entire collection could not be assigned a capsular type, consistent with previous reports [3, 36]. The non-typable isolates might contain a defective *cps* operon not detected using a genotype-based strategy. It is also possible that these few non-typable isolates represent the newly described serotype IX, although the few reported serotype IX isolates were collected from a geographic region distant from our study population [37]. An additional potential limitation of our study is the omission of 20 isolates (2.2%) which had corresponding serotype data but could not be digested and characterized by PFGE. It is possible that exclusion of these isolates could have resulted in a decreased Simpson's diversity index for some of the capsular types. However, given the small fraction of undigested isolates, it is unlikely that their inclusion would significantly alter the relative heterogeneity of capsular types.

In summary, by PFGE, *S. agalactiae* is a genetically diverse organism, with diversity varying within capsular types. Neither *S. agalactiae* capsular-type nor PFGE-type designation alone appear sufficiently discriminatory to determine the relatedness of *S. agalactiae* isolates, given that either typing method alone can link epidemiologically unrelated isolates or fail to identify epidemiologically related isolates. Thus, a combined typing strategy such as PFGE or MLST with capsular typing should be considered to more adequately understand the epidemiology of *S. agalactiae*.

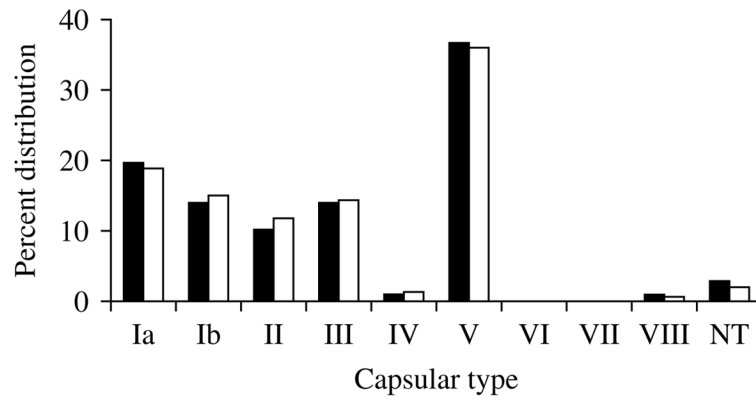
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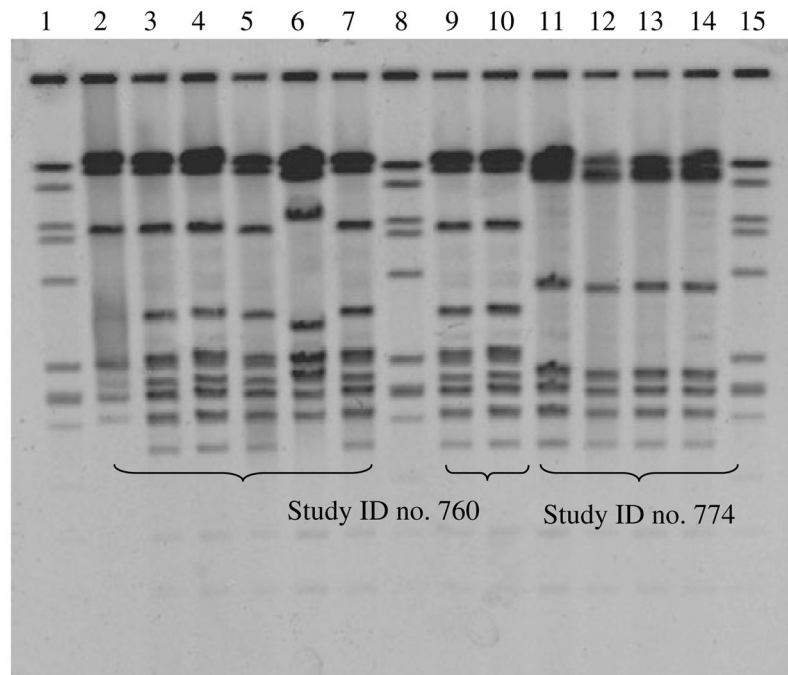
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**Fig. 1.** Capsular-type distribution of *S. agalactiae* isolates collected between January and April 2001, from healthy male and female University of Michigan undergraduates based on the total collection (■,  $n=872$ ) and a subgroup collection of one isolate for each unique PFGE pattern from an individual (□,  $n=168$ ). NT, Non-typable.





**Fig. 2.** Representative PFGE pattern of 12 colonizing *S. agalactiae* isolates recovered from two healthy individuals: Group B *Streptococcus* recovered from throat, rectal and urine samples of study participant no. 760 are shown in lanes 2–7 and 9–10, and from study participant no. 774 in lanes 11–14. Lanes 2–5, 7 and 9–10 from participant no. 760 were capsular type III, while lane 6 was capsular type II. Lanes 11–14 from participant no. 774 were capsular type V. Lanes 1, 8, 15 are control strain A909.

**Table 1**Simpson's diversity index of PFGE within capsular serotypes of *S. agalactiae*

	No. of isolates	Simpson's diversity index	95% CI
Ia	32	0.72	0.546–0.896
Ib	25	0.90	0.832–0.961
II	20	0.93	0.901–0.973
III	24	0.94	0.908–0.982
V	61	0.54	0.391–0.691
All	168	0.92	0.893–0.952

CI, Confidence interval.

Data not shown for capsular types IV ( $n=2$ ) and VIII ( $n=1$ ) and non-typable isolates ( $n=3$ ) [25, 26].