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Biofilm formation of serotype 19 *Streptococcus pneumoniae* clinical isolates in relation to clinical isolate source, pH and Fe(III) supplementation

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ABSTRACT

Aims: *Streptococcus pneumoniae* is one the world's foremost bacterial pathogens that cause massive global mortality and morbidity in young children and immunocompromised adults especially in developing countries. Biofilms have been increasingly recognized as an important prerequisite to disease. Individual *S. pneumoniae* strains differ markedly in their virulence phenotypes, but genetic heterogeneity has complicated attempts to identify any association between a given clonal lineage and propensity to cause a particular disease type. This study investigated serotype 19 *S. pneumoniae* from blood and ear isolates for biofilm formation capacity in relation to isolate source, pH and ferric oxide [Fe(III)] supplementation.
Methodology and results: Viable count and density biofilm assays, microscopy and multi locus sequence typing (MLST) were applied to investigate biofilm formation capacity and genetic diversity of serotype 19 *S. pneumoniae* from blood and ear isolates. Generally, blood isolates were observed to produce more biofilms at both pH 7.4 and 6.8 compared to the ear isolates. The supplementation of Fe(III) was also found to support biofilm growth. Upon MLST typing of the isolates, marked differences in biofilm formation within the same sequence types (ST) of ST199 and ST177 was observed. This strongly indicated that strains within the same sequence type show differences in biofilm formation capacity.
Conclusion, significance and impact of study: This study showed that despite belonging to the same serotype, serotype 19, *S. pneumoniae* blood and ear isolates showed high diversity in biofilm formation ability in relation to pH and Fe(III) supplementation. Additionally, pneumococcal isolates from sequence types ST199 and ST177 also gave rise to differences in biofilm formation ability within the same sequence type (ST). The diversity of biofilm formation within serotype 19 seen in this study is significant to further inform of vaccination strategies against pneumococcal infections, in that due to variations in biofilm formation capacity within the same ST. It is possible that within serotype 19 may show variable vaccination or drug treatment responses. This also indicates that the current treatment strategy which employs specific serotype selection as for PCV14 and PCV7 pneumococcal vaccines may not produce the desired therapeutic results.

Keywords: Biofilms, intra-strain variation, *S. pneumoniae*, genetic diversity

INTRODUCTION

The studies of infectious diseases initiated by Koch and colleagues over 150 years ago were essentially based on the development of pure culture techniques for growth and isolation of planktonic, individual bacterial cells. More recent studies have since shown instead that bacteria predominantly exist in biofilms, as a preferred lifestyle in many natural environments (Fux *et al.*, 2003). Analysis of human biopsy samples and studies in animal models have highlighted the potential importance of biofilm formation by *Streptococcus pneumoniae* during the early stages of colonization and invasion (Hall-Stoodley *et al.*, 2006; Reid *et al.*, 2009; Sanchez *et al.*, 2011).

A large number of bacterial factors have been implicated in pneumococcal biofilm formation in previous studies, including the competence stimulating peptide (CSP) and LuxS quorum sensing systems, as well as various pneumococcal surface structures and virulence factors (Moscoso *et al.*, 2006; Hall-Stoodley *et al.*, 2008; Parker *et al.*, 2009; Trappetti *et al.*, 2009; Sanchez *et al.*, 2011; Trappetti *et al.*, 2011; Vidal *et al.*, 2011; Blanchette-Cain *et al.*, 2013; Shak *et al.*, 2013; Yadav *et al.*, 2013; Pettigrew *et al.*, 2014). However, the link between biofilms

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and disease has been less well defined. While some studies described the importance of biofilms in invasive disease (Trappetti *et al.*, 2011), others have observed that the production of biofilms attenuated disease (Trappetti *et al.*, 2011; Blanchette-Cain *et al.*, 2013; Chao *et al.*, 2014). These differences may have been attributable to the type of experimental models used (Trappetti *et al.*, 2011; Shak *et al.*, 2013).

Many studies involving different *in vitro* models have shown that biofilm formation is significantly affected by the growth medium and other environmental factors, including pH, temperature and osmolarity (Moscoso *et al.*, 2006; Trappetti *et al.*, 2009). Moscoso *et al.* (2006) observed optimal pneumococcal biofilm development when the starting cultures were grown in a semisynthetic (C) medium with pH adjusted in the range 7.0-8.0. A drop in pH to below 6.0 was shown to inhibit the activity of the major autolysin LytA, an important determinant of biofilm formation.

Despite the abundant literature on pneumococcal biofilm formation in clinical isolates, studies on the influence of clinical isolate source and iron availability on biofilm formation have been very limited. These studies include Trappetti *et al.* (2011) who reported that pneumococcal biofilm formation was significantly enhanced in D39 strains in media supplemented with 50 µM Fe(III), while Garcia-Castillo *et al.* (2007) reported that biofilm formation occurred more frequently from cystic fibrosis (CF) respiratory samples than among non-CF blood culture isolates.

Streptococcus pneumoniae cause a myriad of diseases including pneumonia, septicemia, meningitis and otitis media. Additionally, certain serotypes and sequence types (ST) of *S. pneumoniae* have been reported to show a greater potential to cause invasive disease in humans than others. Similarly, pneumococcal strains show variable potential to cause otitis media or otherwise (Hanage *et al.*, 2005; Forbes *et al.*, 2008). This suggests that strains may differ in their capacity to adapt and survive or proliferate within distinct host microenvironments. This further implies that clinical isolates from cases of otitis media (OM) may exhibit distinct *in vitro* and *in vivo* phenotypes from blood isolates. Two conditions that vary significantly between different niches of the human body are metal ion concentrations (McDevitt *et al.*, 2011) and pH; the pH of the blood is typically around 7.4, while the pH of uninfected ear cavity is in the range of 6.5-6.8.

Several recent studies have investigated the relationships of sequence types (ST) in serotype 19 *S. pneumoniae* isolates to cause disease as well as in antimicrobial resistance. In a study on serotype 19F-variants occurring in Brazil, it was observed that 11 out of 154 isolates showed allellic variants of the *wzy* gene was described, comprising ST810 and ST1363 of varying antimicrobial patterns. Although sequences of the *wzy*19F gene of these variants were identical to each other and to those previously described in Brazil, they were different from *wzy*19F variants identified in other countries (Oliviera *et al.*, 2021). In a systematic literature review to analyse the incidence, García *et al.* (2021) found that clonal distribution in serotype 19A was heterogeneous within countries and regions, irrespective of the vaccine used; and the diversity of 19A isolates increased after vaccination. It was associated with frequent serotype switching events and with the prevalence of multidrug resistant strains. In a study on macrolide resistance in the Czech Republic, via MiSeq sequencing and core genome multilocus sequence typing (cgMLST), Spanelova *et al.* (2020) showed that 58 serotype 19A *S. pneumoniae* isolates belonging to the macrolide resistant ST416 showed serotype switch between 15B and 19A.

Streptococcus pneumoniae is an immensely diverse bacterial species comprising more than 90 different serotypes according to the capsule differences. Adding to that there are over 4000 different sequence types of *S. pneumoniae*. As a consequence of this diversity, some serotypes show higher prevalence of the diseases caused. A study by Trappetti *et al.* (2013) and Amin *et al.* (2015) showed that serotype 3 and 14 pneumococcal isolates showed different biofilm formation capacity depending on source of isolate (blood vs ear source), pH and supplementation of ferric oxide (Fe[III]). This study focused on serotype 19 pneumococcal isolates as some studies have demonstrated a high genetic diversity in serotype 19F and 19A isolates. This study aimed to investigate if indeed like serotype 3 and 14, biofilm formation capacity is also differently seen between blood and ear sourced isolates when they are grown in different pH and Fe(III) supplementation. Further understanding of genetic differences in serotype 19 *S. pneumoniae* biofilm formation may help to understand the information of genetic diversity in pneumococci when exploring drug and other treatment strategies.

MATERIALS AND METHODS

Clinical isolates

A total of 10 *Streptococcus pneumoniae* clinical isolates (blood, n=5; ear, n=5) of serotype 19 were investigated for their ability to form biofilm *in vitro* by the biofilm assay. All strains (blood strains – KPJ10, KPJ11, KPJ12, KPJ13, KPJ14 and ear strains – KPJ15, KPJ16, KPJ17, KPJ18, KPJ19) were acquired from KPJ Hospital Kota Kinabalu Sabah, Malaysia.

Biofilm assay for growth (CFU/mL), density (Abs_{570}) counts and microscopic analysis

Glycerol bacterial stocks were firstly grown on blood agar overnight at 37 °C in 5% CO_2. The colonies were precultured in 1.0 mL C+Y media in pH 7.4 and 6.8 at an OD_{600} of 0.05 and incubated to a final OD_{600} of 0.3. The cultures were then diluted 1/100 in phosphate buffer saline (PBS, pH 7.0) and 180 µL of the diluted suspension was transferred into wells of a Costar (Corning) flat-bottomed 96 well microtiter plate with or without the addition of 100 µM Fe(III). The biofilm assays were carried out in triplicates in two independent experiments

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Table 1: Primer sequences used for MLST typing.

Primer	Sequence (5'-3')
recP (transketolase)	
recP F	GCCAACTCAGGTCATCCAGG
recP R	TGC AAC CGT AGC ATT GTA AC
aroE (shikimate dehydrogenase)	
aroE F	GCC TTT GAG GCG ACAGC
aroE R	TGC AGT TCA (G/A)AA ACA T(A/T) T TCT AA
ddl (D-alanine ligase)	
ddl F	TGC (C/T)CA AGT TCC TTA TGT GG
ddl R	CAC TGG GT(G/A) AAA CC(A/T) GGC AT
gdh (glucose-6-phosphate dehydrogenase)	
gdh F	ATG GAC AAA CCA GC(G/A/T/C) AG(C/T) TT
gdh R	GCT TGA GGT CCC AT(G/A) CT(G/A/T/C) CC
gki (glucose kinase)	
gki F	GGC ATT GGA ATG GGA TCA CC
gki R	TCT CCC GCA GCT GAC AC
spi (signal peptidase I)	
spi F	TTA TTC CTC CTGA TTC TGT C
spi R	GTG ATT GGC CAG AAG CGG AA
xpt (xanthine phosphoribosyltransferase)	
xpt F	TTA TTA GAA GAG CGC ATC CT
xpt R	AGA TCT GCC TCC TTA AAT AC

and incubated at 37 °C in a 5% CO_2 incubator for 24 h respectively. For the quantification of bacteria count attached on the microtiter plates, the plates were washed with Luria Bertani (LB) medium three times after incubation. Upon final wash, a volume of 100 µL of fresh LB media were added into the wells. The plates were then sealed with parafilm and sonicated at 35 kHz for 3 sec in a Transonic 460/H sonicating water bath. The dispersed biofilm derived bacterial suspension was then serially diluted before being plated on to blood agar plates and incubated at 37 °C in 5% CO_2 overnight. Upon incubation, the colonies were counted (CFU/mL).

For the microscopic examination of biofilms, wells of microtiter plates were washed three times in LB medium to remove planktonic bacteria. The wells were dried in a 37 °C incubator for 30 min and stained with 0.5% crystal violet for 30 min in a 37 °C incubator. Crystal violet was then removed from each well and the plate was left at room temperature for 10 min. The wells were then washed three times in PBS and observed under transmitted light using an AMG Evos inverted microscope under 40× objective. Assays were carried out in triplicates for each sample.

For the quantitation of biofilm density, 50 µL absolute ethanol was dispensed into the wells of the crystal violet-stained microtiter plates and measured for the absorbance at 570 nm using a Spectromax M2 spectrophotometer (Molecular Devices). All assays were performed in replicates and repeated three times at day 1, day 3 and day 6.

Multi-locus sequence typing (MLST) of the clinical isolates

DNA from both blood and ear isolates were extracted using the Qiagen DNAEasy Blood and Tissue Kit according to manufacturers' instructions and subjected to PCR under the following conditions: 30 cycles of amplification of 95 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 72 °C for 1 min (extension). The primer sequences (Enright and Spratt, 1998) used are listed in Table 1.

The samples were then sequence analysed for portions of the conserved housekeeping genes *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*. Sequencing of samples were carried out by ScienceVision Sdn Bhd. Results received were then compared against the MLST database (http://www.pubmlst.org) for identification of the respective sequence types (ST) of strains based on the resulting allelic profiles of the isolates sequenced. The determination of sequence types of strains was used to indicate the similarity of ST distributions for blood and ear isolates for each serotype.

RESULTS

Influence of isolate source, pH and Fe(III) supplementation on pooled isolate *in vitro* biofilm formation

In reference to the influence of isolate source on biofilm formation, as seen in Figure 1, blood isolates demonstrated a higher ability in forming biofilm than ear isolates in the two studied conditions at pH 7.4 and pH

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6.8. At pH 6.8, the blood isolates grown in C+Y medium only (without Fe[III]) also formed significantly better in vitro biofilm formation (p<0.01) than ear isolates. Significant increases were observed for blood isolates when grown without Fe(III) supplementation compared to ear isolates at pH 7.4 (p≤0.001) and pH 6.8 (p≤0.01). As seen in Figure 2, when referring to isolate source comparison, the crystal violet biofilm assay which is classically used for staining of biofilms showed that the Abs_{570} readings showed no significant differences in biofilm formation between the two isolate types for both pH 7.4 and pH 6.8.

In regard to pH influence, Figure 1 showed that biofilm growth of all strains were generally better at pH 7.4 compared to pH 6.8, with significantly better biofilm formation seen at pH 7.4 for blood isolates without Fe(III) supplementation (p≤0.001). Serotype 19 ear isolates did not exhibit any significant differences between the two pH. In Figure 2, via the crystal violet assay, it can be seen that biofilm formation for blood isolates at pH 7.4 without Fe(III) was also similar to Figure 1, where significantly better biofilm growth capacity was achieved at pH 7.4 compared to pH 6.8. Therefore, for biofilm formation of blood isolates at pH 7.4 without Fe(III) it can be said that results in Figure 1 for the viable count assay concurred with that of Figure 2 for the crystal violet assay.

As for the influence of Fe(III) supplementation, as seen in Figure 1, the significant positive influence of iron on biofilm growth *in vitro* was not observable for all isolates at both pH. This observation was also concurred by the crystal violet biofilm assay in Figure 2. Since better biofilm growth was observed at pH 7.4 for both isolate types, 1 blood (KJP10) and 1 ear isolate (KPJ15) was randomly selected from each serotype for microscopic observation to view the influence of Fe(III) in biofilm formation.

In a stark contrast to the above findings, microscopic examinations as seen in Figure 3, the blood and ear sourced clinical isolates revealed a physical evidence of the positive influence of Fe(III) supplementation; where at pH 7.4 with the addition of Fe(III) increased biofilm growth for all isolate types. The microscopic images of serotype 19 blood isolate KPJ10 in C+Y appeared to show more marked bacterial aggregation at pH 7.4 [Figure 3 (i)] than at pH 6.8 [Figure 3 (iii)], while supplementation with Fe(III) increased bacterial aggregation at only pH 6.8 [Figure 3(iv)]. At pH 7.4, ear isolate appeared to show increased biofilm formation in the presence of Fe(III) [Figure 3(vi)] compared to without Fe(III) [Figure 3(v)]. When grown in C+Y+Fe(III) at pH 6.8 (viii), the strain also displayed visibly reduced aggregation compared to pH 7.4 [Figure 3(vi)].

Genetic diversity analysis of serogroup 19 clinical isolates by MLST typing

Since a direct disconnect was observed between the microtiter plate assays and microscopic analysis of Fe(III) supplemented biofilms at pH 7.4, MLST typing were performed on 9 isolates of serotype 19 from blood and ear (Table 2) and biofilm growth counts were determined singly (Figure 4) to determine if the disconnect in observations were due to intrastrain variations between each isolate which we postulated would give rise to the high variability of growth of biofilms. Interestingly, it was found that even between the clonally identical strains of ST 199 blood isolates (KPJ11 and KPJ13) and ST177 ear isolates (KPJ16 and KPJ19) (Table 2), their capacity to form biofilms were also distinct within the same clonal groups (Figure 4). KPJ11/ST199 blood isolate was observed to be a significantly better biofilm former than KPJ13/ST199 blood isolates at pH 7.4 (p≤0.001), while ear isolate KPJ16/ST177 formed significantly (p≤0.01) better biofilm former at pH 6.8 than ear isolate KPJ19/ST177.

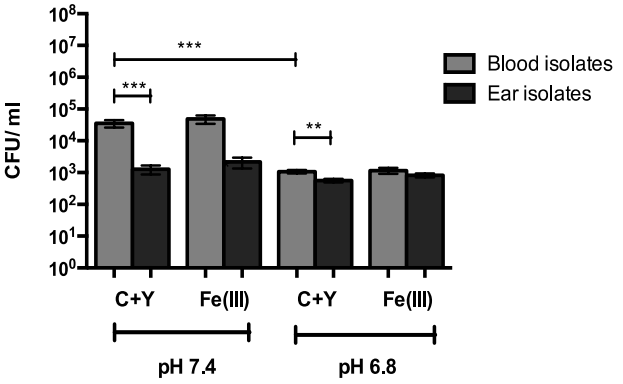

Figure 1: Biofilm formation by *Streptococcus pneumoniae* serotype 19 clinical isolates (5 from blood and 5 from ear) after 24 h of growth at either pH 7.4 or 6.8, with or without 100 µM Fe(III), determined by viable count (CFU/mL). Data are the means ± standard deviations for three independent experiments (*, p<0.05; **, p<0.01 and ***, p<0.001; 2-tailed Student's t-test).

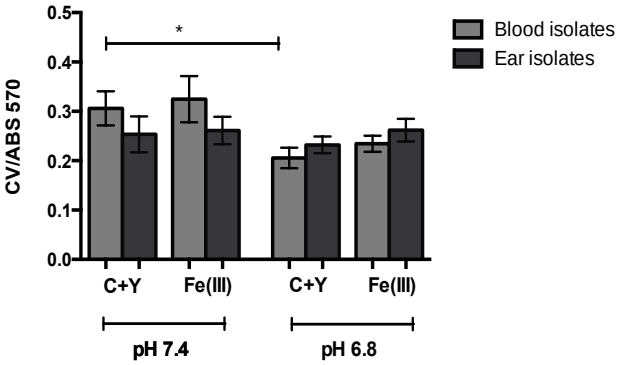

Figure 2: Biofilm formation by *Streptococcus pneumoniae* serotype 19 clinical isolates (5 from blood and 5 from ear) after 24 h of growth at either pH 7.4 or 6.8, with or without 100 µM Fe(III), determined by density counts (ABS_{570}). Data are the means ± standard deviations for three independent experiments (*, p<0.05; **, p<0.01 and ***, p<0.001; 2-tailed Student's t-test).

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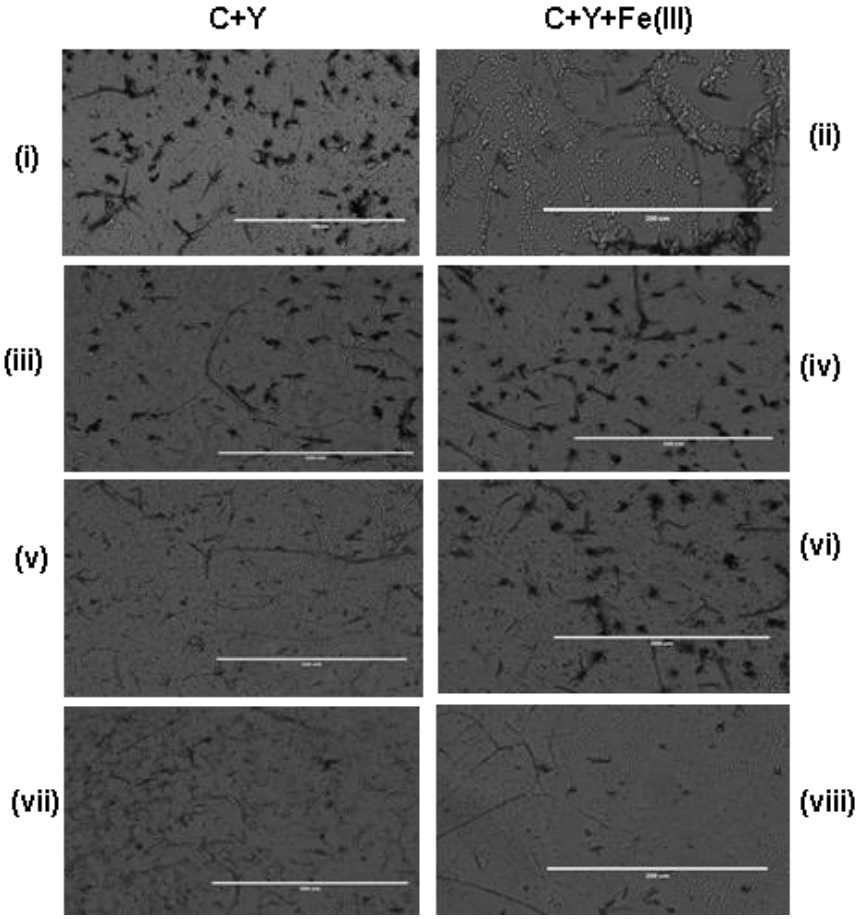

Figure 3: Microscopic analysis of crystal violet-stained 24 h biofilms of serogroup 19 blood and ear isolates. (i) Blood isolate KPJ10 grown in C+Y media at pH 7.4; (ii) Blood isolate KPJ10 grown in C+Y media with 100 µM Fe(III) at pH 7.4; (iii) Blood isolate KPJ10 grown in C+Y media at pH 6.8; (iv) Blood isolate KPJ10 grown in C+Y media with 100 µM Fe(III) at pH 6.8; (v) Ear isolate KPJ15 grown in C+Y media at pH 7.4; (vi) Ear isolate KPJ15 grown in C+Y media with 100 µM Fe(III) at pH 7.4; (vii) Ear isolate KPJ15 grown in C+Y media at pH 6.8; (viii) Ear isolate KPJ15 grown in C+Y media with 100 µM Fe(III) at pH 6.8. Scale bar = 0.2 mm; 40× magnification).

Table 2: Identification of Sequence-type (ST) of serotype 19 clinical isolates by MLST typing.

Serotype 19 strain	MLST type
KPJ10 (Blood isolate)	19F/876
KPJ11 (Blood isolate)	19F/199
KPJ12 (Blood isolate)	19F/186
KPJ13 (Blood isolate)	19F/199
KPJ15 (Ear isolate)	19F/1718
KPJ16 (Ear isolate)	19F/177
KPJ17 (Ear isolate)	19F/309
KPJ18 (Ear isolate)	19F/2417
KPJ19 (Ear isolate)	19F/177

DISCUSSION

The formation of biofilms has been implicated in many studies as an important prerequisite for pneumococcal disease (Moscoso *et al.*, 2006; Marks *et al.*, 2012; Pettigrew *et al.*, 2014). Therefore, it is important to understand the molecular determinants that regulate pneumococcal biofilm formation, particularly in the host environment, where pneumococci may encounter adverse environments such as antibiotics and host immune responses. This study aimed to investigate the role of clinical isolate source and Fe(III) supplementation in biofilm formation by 10 blood and ear isolates of *S. pneumoniae* belonging to serogroup 19, which were grown at either pH 7.4 or pH 6.8 to reflect the respective physiological conditions in the blood and ear niches (McDevitt *et al.*, 2011). Biofilm formation assay in Figures 1, 2 and 4 showing that most isolates formed biofilms appeared to concur with the findings of Camilli *et al.*

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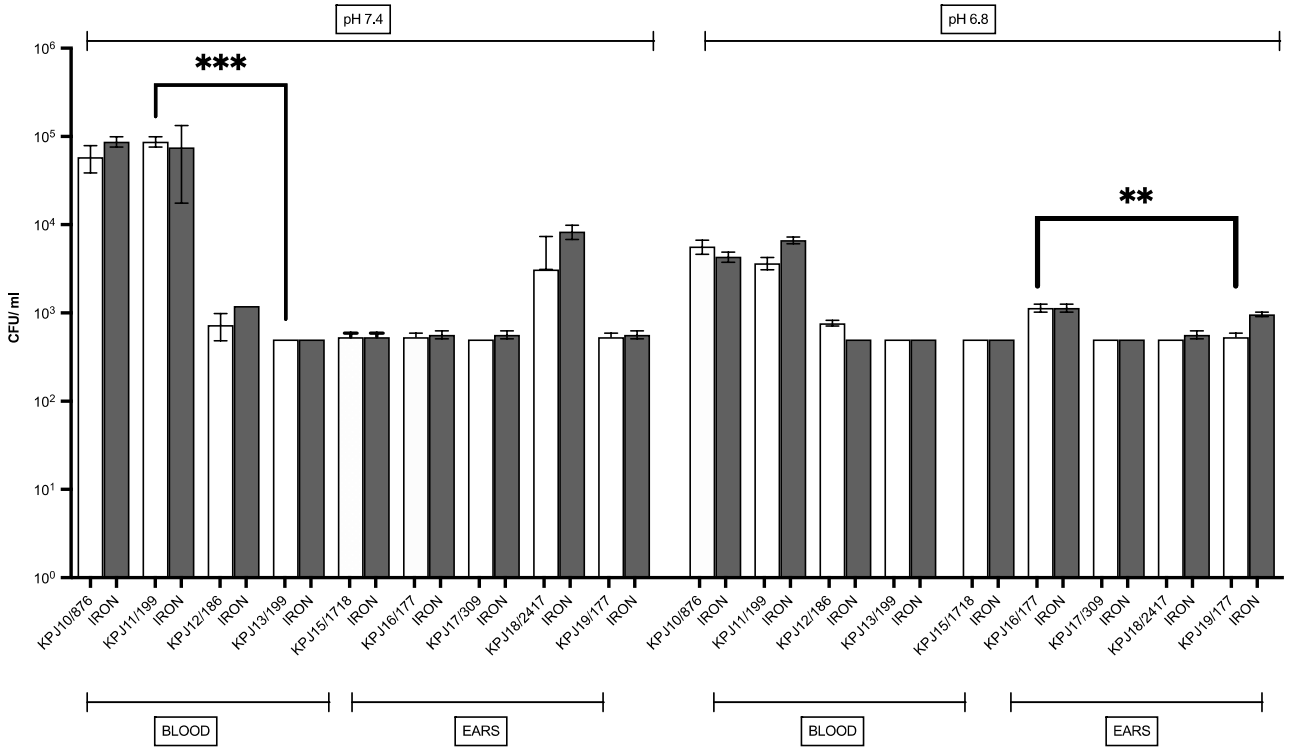

Figure 4: Sequence-type (ST) identification of Serotype 19 blood and ear isolates (4 from blood and 5 from ear) at either pH 7.4 or 6.8, with 100 µM Fe(III) (grey bars) or without 100 µM Fe(III) (white bars), determined by MLST typing. Data are the means ± standard deviations for three independent experiments *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$; 2-tailed Student's *t*-test).

(2011) and others (Allegrucci *et al.*, 2006; Garcia-Castillo *et al.*, 2007; Chao *et al.*, 2014; Domenech *et al.*, 2014) that biofilm formation is a common feature among pneumococcal strains. When investigating the influence of Fe(III) supplementation on biofilm formation some strain variations were also observed, but generally biofilm formation of most blood and ear isolates was enhanced in the presence of Fe(III) at pH 7.4. This agreed with Trappetti *et al.* (2011) who found that biofilm formation of the type 2 strain D39 was significantly enhanced when supplemented with Fe(III) at pH 7.4. An interesting finding in this study was that at pH 6.8 Fe(III) supplementation did not result in significantly increased biofilm formation in the serogroup 19 isolates. In general, despite high strain-strain variation in biofilm formation by blood and ear isolates seen in Figure 4, the viable count assays in Figure 1 indicated that differences in pH could influence biofilm formation capacity of the blood isolates. Blood isolates of serogroup 19 in C+Y media without Fe(III) showed significant increases in biofilm formation at pH 7.4 relative to pH 6.8 ($p<0.001$), while ear isolates did not show significant biofilm formation increases at pH 6.8 compared to pH 7.4.

In general, as can be seen in Figure 3, the positive influence of Fe(III) in biofilm formation was indicated in blood isolates at only pH 6.8. Blood isolates showed no differences in biofilm formation when comparisons were made between C+Y only and C+Y+Fe(III) at pH 7.4. Furthermore, the microscopic images of ear isolates in Fe(III) at pH 7.4 showed markedly higher aggregations of biofilms, while at pH 6.8 the effect of Fe(III) for the ear isolates was either insignificant or inhibitory. This seems to suggest that biofilm formation capacity of these serotype 19 bacterial isolates are niche (blood or ear sourced) dependent (tissue tropism). Studies by Amin *et al.* (2015) and Trappetti *et al.* (2013) have also shown the phenomenon of tissue tropism in biofilm formation capacity of pneumococcal isolates in Serotypes 14 and 3, respectively.

An important challenge in this study was the occurrence of marked variability in biofilm formation between isolates, which complicated interpretation of findings regarding the influence of clinical source, Fe (III) or pH on *in vitro* biofilm formation. Serogroup 19 comprises several structurally-related, immuno-cross-reactive capsular types (19A, 19B, 19C and 19F), which may further contribute to genetic diversity within this group. The marked variation in biofilm formation phenotype between strains from different clinical sources, and the variable influence of pH and Fe(III) supplementation may well reflect this diversity as seen in this study, where out of 9 isolates 7 distinct STs were identified. Indeed, marked variation in biofilm formation capacity among individual pneumococcal strains has

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been demonstrated by others (Tapiainen *et al.*, 2010; Camilli *et al.*, 2011). Genetic diversity is a characteristic feature of *S. pneumoniae* with 93 different serotypes superimposed on over 5000 clonal lineages recognizable by MLST (Enright and Spratt, 1998). Thus, much of the strain-strain variation in biofilm phenotype might be attributable to the presence of multiple STs within a serotype/group. As seen in this study, significant differences in biofilm formation capacity was observed in ST199 blood isolates as well as ST177 ear isolates. This study concurred that of Rockett *et al.* (2018) where in a genomic analysis of 124 serotype 19 pneumococcal isolates, 35 different sequence types were identified which were predominated (78/124) by ST199, ST320, ST63 and ST2345.

CONCLUSION

The work described in this study has identified marked discrepancies between the influence of Fe(III), pH and isolate source on biofilm phenotype in the serogroup 19 clinical isolates and ST types. It has also identified intra-strain variation of *S. pneumoniae* isolates within the same serogroup and ST type. This is a significant study to understand genetic diversity of serotype 19 *S. pneumoniae* and important to further investigate these differences using *in vivo* models to determine whether they have relevance in terms of pathogenesis of pneumococcal disease.

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CONFLICT OF INTEREST

The authors declare that the research was carried out in the absence of any commercial or financial relationships, therefore, conflict of interests does not exist.

INFORMED CONSENT

There were no animals involved in this study.

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