In vitro bactericidal activity of Streptococcus pneumoniae and susceptibility of Staphylococcus aureus strains isolated from co-colonized vs. non-co-colonized children

Gili Regev-Yochay¹,², Richard Malley², Ethan Rubinstein³, Meir Raz⁴, Ron Dagan⁵ and Marc Lipsitch¹.

¹Department of Epidemiology and Department of Immunology & Infectious Diseases, Harvard School of Public Health, Boston, MA. USA, ²Division of Infectious Diseases, Department of Medicine, Children's Hospital Boston, Harvard Medical School, Boston, MA. USA, ³Infectious Diseases Section University of Manitoba, Winnipeg, Manitoba, Canada ⁴Hashfela District, Macabbi Healthcare Services, Rishon-Lezion, Israel, ⁵Pediatric Infectious Disease Unit, Soroka University Medical Center, and the Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

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**ABSTRACT**

*Streptococcus pneumoniae* is bactericidal to *Staphylococcus aureus* *in vitro*. To determine whether this *in vitro* effect accounts for the inverse relation between *S. pneumoniae* and *S. aureus* colonization reported in previous epidemiologic studies, we compared *S. pneumoniae* and *S. aureus* strains from co-colonized children to those from non-co-colonized children. Co-colonizing pneumococci were less bactericidal and co-colonizing staphylococci less susceptible to this effect; however, the magnitude of the effect was small. Thus, *in vitro* killing is not the major determinant of the pattern of co-colonization.
Streptococcus pneumoniae and Staphylococcus aureus are common causes of community acquired infections and account for significant morbidity and mortality worldwide (3-6). Nasal S. aureus and nasopharyngeal S. pneumoniae colonization serve as the major source for person-to-person spread and as the source for endogenous invasive disease (1, 15).

Several epidemiologic studies have demonstrated an inverse relationship between S. aureus and S. pneumoniae carriage in children (2, 7, 10, 14). These findings have raised concerns that widespread use of pneumococcal conjugate vaccines may lead to an increased incidence of S. aureus morbidity (13). We recently studied the direct in vitro effect of S. pneumoniae on S. aureus, using a few laboratory and clinical strains and their variants. We have reported that S. pneumoniae is bactericidal to S. aureus, and that the bactericidal factor is hydrogen peroxide (H₂O₂) (11). We observed that different streptococcal species exhibit a variable bactericidal activity and that different staphylococcal species exhibit variable susceptibility to this effect.

The aim of this study was to assess whether variability in bactericidal activity of S. pneumoniae strains and/or variability in susceptibility to this effect by S. aureus strains could predict their pattern of S. aureus and S. pneumoniae co-colonization. All strains tested in this study were collected in our previously reported epidemiologic study that assessed the association between nasopharyngeal carriage of S. pneumoniae and nasal carriage of S. aureus (10). In the original study, nasopharyngeal and nasal swabs were separately obtained from children aged 40 months or younger, on a single occasion. The study population was not vaccinated against S. pneumoniae, as the pneumococcal conjugate vaccine was not registered in Israel at the time of the study. Nasopharyngeal
swabs were streaked onto tryptic soy agar plates supplemented with 5% sheep blood and 5µg/ml gentamicin. Colonies that were α-hemolytic and susceptible to optochin were identified as *S. pneumoniae*. Nasal swabs were streaked on tryptic soy agar plates supplemented with 5% sheep blood. *S. aureus* was identified by morphology, β-hemolysis, catalase, DNAase and coagulase. All isolated strains were stored at -80°C.

For the present study, we randomly picked 27 *S. pneumoniae* strains isolated from co-colonized children and 30 strains isolated from children colonized only with *S. pneumoniae* (10). We compared the mean bactericidal effect of the two groups of strains. To quantify the bactericidal effect, we followed a method described previously (11). Briefly, all strains were initially grown to late logarithmic phase (OD: 0.4-0.8), stored at -80°C and thawed on the experiment day. The bactericidal effect of each pneumococcal strain was tested on *S. aureus* strain Newman (NCTC 8178). Serial two-fold dilutions of the staphylococcal strain (beginning with 1×10^8 CFU/ml) were mixed with serial two-fold dilutions of the streptococcal strain (beginning with 4×10^7 CFU/ml) in a final volume of 100 µl. Cultures were incubated at 37°C in 5% CO₂. After 6 hours of incubation, approximately 2 µl of each culture were plated using a replica plater (Sigma-Aldrich) on selective media; tryptic soy agar supplemented with 5% sheep blood and 8µg/ml gentamicin for *S. pneumoniae* and mannitol-salt agar for *S. aureus*. The log₁₀ of the maximum inoculum of this strain “killed” (reduced below the detection limit of 50 CFU/well) by 10^6 CFU of streptococci at 6 hours is reported as the IK₆.

Similarly, to assess whether *S. aureus* strains carried by co-colonized children were less susceptible to the pneumococcal cidal effect, we compared strains from co-colonized children to strains from children colonized only with *S. aureus*, using the same
protocol as described above. In this experiment, the reference *S. pneumoniae* strain used was TIGR4 (12), against which all *S. aureus* strains were tested. During the experiments the investigator was blinded to the colonization status of the strains tested (co-colonized or not).

Since we observed between-day variation in IK₆ results for the same strain, we adjusted for this variation in both the *S. aureus* and the *S. pneumoniae* comparisons by using a mixed-effects model in which “day” was incorporated as a random effect variable.

*Streptococcus pneumoniae* strains from children co-colonized with *S. aureus* (n=27) showed a slightly lower mean bactericidal effect (IK₆=6.72±0.12) than those from non-co-colonized (n=30) (IK₆=6.98±0.09, p=0.051). *S. aureus* strains from children co-colonized with *S. pneumoniae* strains (n=25) were slightly more resistant to the bactericidal effect (IK₆ = 6.70±0.09) than those from non-co-colonized strains (n=24) (IK₆=6.81±0.08, p=0.12). Thus, in both cases the difference was small and the effect did not reach statistical significance. Confidence intervals (95% CI) for the difference in IK₆ (co-colonizers vs. single colonizers) were -0.34 to 0.08 for *S. pneumoniae* and -0.20 to 0.01 for *S. aureus*, suggesting at most a modest difference of about 2-3 fold killing in 6 hours between the two groups (Figure 1a-b).

We next hypothesized that the strains carried by co-colonized children should include either a highly resistant *S. aureus* strain or a less bactericidal *S. pneumoniae* strain, or possibly both. To examine this hypothesis we tested 41 pairs of *S. aureus* and *S. pneumoniae* strains that were simultaneously isolated from co-colonized children and assessed whether there is a negative correlation between the susceptibility of a co-
colonizing *S. aureus* strain and the bactericidal effect of the co-colonizing *S. pneumoniae* strain from the same subject. To adjust for the daily variation, in this experiment we included a specific strain that was examined every day and the results were standardized according to its daily variation. Although a trend towards a negative correlation (consistent with our hypothesis) was observed, the correlation was low and non-significant (Spearman $\rho = -0.184$, $p = 0.249$) (Figure 2).

Our study suggests that results of the *in vitro* evaluation of the bactericidal effect of *S. pneumoniae* and susceptibility of *S. aureus* are not major predictors of the epidemiologic pattern of co-colonization. While in all experiments we did observe a trend that was consistent with our hypothesis (i.e., strains from co-colonized children were less bactericidal (for *S. pneumoniae*) or less susceptible (for *S. aureus*)), this trend was nearly statistically significant only for the *S. pneumoniae* strains, and the effect was small in all cases. A larger study would have perhaps yielded narrower confidence intervals, and thus defined this modest effect as statistically significant, but since the 95% confidence intervals defining the difference are already narrow relative to the variability in each population, we suggest that variability in the *in vitro* bactericidal effect probably has at most a minor role in determining the pattern of co-colonization. Our findings are also consistent with those of Melles et al. who failed to find an association between bacterial genotypes and the pattern of co-colonization (8).

The discrepancy between the strong *in vitro* interference (11) and the minor differences observed between co-colonizing and non-co-colonizing strains has several
potential explanations. First, we might have misclassified "co-colonizing strains" due to our original epidemiologic study design, which assessed point prevalence. Because we could not assess the dynamics of colonization, we have no way of knowing whether one of the strains found as co-colonizers on the day of sampling might have been about to be cleared from that host, or on the other hand, whether a strain found without the other species might have been found co-colonizing a day later. As a result, strains with the ability to co-colonize may have been detected as non-co-colonizers on the particular day of sampling, while strains that tended to inhibit or be inhibited by the other species may have transiently been co-colonizing on the day of sampling. Thus, the point-prevalence design may have resulted in "misclassification" of the ability of any given strain to co-colonize, tending to bias findings toward the null. Second, the strong in vitro effect of \( \text{H}_2\text{O}_2 \) may have only a subtle role in the in vivo interference and host immune factors may play a more significant role in vivo. The recent report by McNalley et al. (7) which shows that the negative association between \( S. \text{pneumoniae} \) and \( S. \text{aureus} \) carriage observed in non-HIV children does not exist in HIV positive children suggests that the association between \( S. \text{pneumoniae} \) and \( S. \text{aureus} \) requires an intact host immune response. The fact that \( S. \text{aureus} \) and \( S. \text{pneumoniae} \) often colonize different parts of the upper respiratory tract (1, 9) may also be suggestive of a host immune response mechanism rather than a direct pathogen interference mechanism.

Although our study suggests that the variation in \( \text{H}_2\text{O}_2 \) production does not explain the pattern of co-colonization, it does not exclude that \( \text{H}_2\text{O}_2 \) may be necessary for the interference to occur, whether through direct bacterial interference mechanism, or through an indirect host response mechanism. Even if \( \text{H}_2\text{O}_2 \) were directly responsible for
this interference, the degree of variation in production (S. pneumoniae) and susceptibility (S. aureus) might be too limited to be a major determinant of the pattern of co-colonization.

Both mechanistic and epidemiological studies are required to further elucidate the mechanism of in vivo interference between S. aureus and S. pneumoniae and to determine the implications of the wide use of pneumococcal conjugate vaccine on S. aureus colonization and infections.
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Legends to Figures:

Figure 1: (a) *S. pneumoniae* bactericidal effect, and (b) *S. aureus* susceptibility to the *S. pneumoniae* effect, as measured by IK₆. Strains from co-colonized children (squares) compared to strains from non-co-colonized children (triangles). The reference *S. aureus* strain used in (a) is *S. aureus* Newman. The reference *S. pneumoniae* strain used in (b) is TIGR4.

Figure 2: The correlation between the *S. pneumoniae* IK₆ and the *S. aureus* IK₆ of pairs of co-colonizing bacteria. Dotted line is the 95%CI of the fitted correlation line.
REFERENCES


Figure 1:

A: S. pneumoniae strains

B: S. aureus strains

log_{10} CFU killed (IK6)

Co-colonized Not Co-colonized

p = 0.051

p = 0.12
Figure 2:

S. pneumoniae vs S. aureus IK₆ correlation.