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Streptococcus pyogenes colonization in children aged 24-59 months in The Gambia: Impact of Live Attenuated Influenza Vaccine and associated serological responses

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Background: Immunity to *Streptococcus pyogenes* in high burden settings is poorly understood. We explored *S. pyogenes* nasopharyngeal colonization after intranasal live attenuated influenza vaccine (LAIV) among Gambian children aged 24-59 months, and resulting serological response to 7 antigens.

Methods: A post-hoc analysis was performed in 320 children randomized to receive LAIV at baseline (LAIV group) or not (control). *S. pyogenes* colonization was determined by quantitative Polymerase Chain Reaction (qPCR) on nasopharyngeal swabs from baseline (D0), day 7 (D7) and day 21 (D21). Anti-streptococcal IgG was quantified, including a subset with paired serum pre/post *S. pyogenes* acquisition.

Results: The point prevalence of *S. pyogenes* colonization ranged from 7-13%. In children negative at D0, *S. pyogenes* was detected at D7 or D21 in 18% of LAIV group and 11% of control group participants (p=0.12). The odds ratio (OR) for colonization over time was significantly increased in the LAIV group (D21 vs D0 OR 3.18, p=0.003) but not in the control group (OR 0.86, p=0.79). The highest IgG increases following asymptomatic colonization were seen for M1 and SpyCEP proteins.

Conclusions: Asymptomatic *S. pyogenes* colonization appears modestly increased by LAIV, and may be immunologically significant. LAIV could be used to study influenza-*S. pyogenes* interactions.

Keywords: *Streptococcus pyogenes*, Colonization , Carriage , The Gambia, Live Attenuated Influenza Vaccine, Serological responses, Antibodies

INTRODUCTION

Streptococcus pyogenes (Group A Streptococcus, Strep A) is responsible for half a million deaths worldwide each year, mainly in low- and middle-income countries (LMIC), through severe invasive infections and immune complications including rheumatic heart disease [1, 2]. Development of a vaccine against *S. pyogenes* was declared a global health research priority in 2018 by the World Health Assembly. An ideal vaccine would substantially reduce *S. pyogenes* transmission, disease, and perhaps colonization, without provoking immune mediated complications [3]. Observational data demonstrate decreased *S. pyogenes* incidence with increasing age, possibly explained by naturally acquired immunity[4]. Estimated point prevalence of asymptomatic pharyngeal colonization with *S. pyogene*s is 6.6-9.7% in children [4]. Characterizing protective immunity generated by infections and colonization remains a major knowledge gap in designing effective and safe vaccines, particularly from LMIC settings [5-7].

Epidemiological observations have demonstrated association between invasive *S. pyogenes* infections and respiratory viral infections, especially influenza [8]. *S. pyogenes* was responsible

for substantial mortality in influenza pandemics of 1918 and 2009 [8, 9]. It is important to understand whether respiratory viruses increase colonization with pathogenic bacteria such as *S. pyogenes* in the nasopharynx, as colonization may be a necessary preceding event to pharyngitis and invasive disease. We have recently demonstrated that intranasal live attenuated influenza vaccine (LAIV), used as a surrogate viral challenge agent, induces modest increases in *Streptococcus pneumoniae* colonization and density in the 21 days following vaccination in Gambian children [10]. We conducted a post-hoc analysis of this randomized controlled trial of LAIV in children aged 24-59 months to explore whether LAIV increased nasopharyngeal *S. pyogenes* colonization, and whether colonization was associated with a serological response to several *S. pyogenes* antigens.

METHODS

Study population and design: We conducted a post-hoc observational study nested within a randomized controlled trial of LAIV, studying immunogenicity, viral shedding and microbiome interactions in children aged 24-59 months in Sukauta, an urban region of The Gambia. The study (NCT02972957) was conducted over two years between February to April 2017, and January to March 2018 [10, 11]. Children were randomized 2:1 to receive LAIV at study entry (day 0, D0, LAIV group) or on day 21 (D21, control group), which was the end of active follow up. All participants were influenza-vaccine naïve and clinically well, with no history of respiratory illness in the prior 14 days. Participants in LAIV and control groups were recruited simultaneously to avoid bias via seasonal variation. Baseline respiratory virus status was determined with multiplex PCR[10]. Children were immunized with the Northern Hemisphere Russian-backbone Trivalent LAIV (Nasovac-S, Serum Institute of India, Pune, India)[11]. Data on symptoms experienced between D0 and D7 were collected at the D7 visit, and symptoms experienced between D7 and D21 recorded at the D21 visit. The study was approved by the Joint Medical Research Council/Gambia Government ethics committee (ref: 16193). Informed consent was obtained from all parents, including for subsequent research on their samples..

Determination of *S. pyogenes* colonization status: Participants had a nasopharyngeal swab taken at D0 (immediately prior to LAIV receipt), at D7 and D21, using flocked swabs (FLOQSwabs; Copan, USA) stored in RNAprotect (Qiagen, UK). Samples were processed within 4 h of collection and stored at −70°C until further processing. DNA was extracted from 200µl of RNAprotect using the AGOWA Mag Mini DNA extraction kit (LGC Genomics, Berlin, Germany) in combination with phenol-bead beating as previously described[12]. Standard curves were generated via extraction of total genomic DNA from the *S. pyogenes* H293 reference strain, ranging from 1 to 10,000,000 genome copies per µL. Quantitative PCR was performed using QuantStudio™ 5 Real-Time PCR System, using primers and probes to detect the *S. pyogenes* specific gene *speB* (Forward: CTAAACCCTTCAGCTCTTGGTACTG, Reverse: TTGATGCCTACAACAGCACTTTG, Probe: Cy5-CGGCGCAGGCGGCTTCAAC-BHQ2)

[13]. A cycle threshold of 40 was defined as positive, but all curves above CT threshold of 35 were checked manually to ensure appearance was consistent with true amplification of the target. Samples were run in triplicate. Where only one of the triplicates showed the presence of *S. pyogenes*, samples were repeated and only defined as positive if at least two triplicates were positive on repeat testing. A participant with positive qPCR result at a given timepoint was considered colonized. All participants were included in the colonization study group. Participants who were negative at baseline were included in the acquisition study group (figure 1).

Antibody measurement: Blood was collected in serum separation tubes from participants in the LAIV group, for study endpoints on influenza vaccine immunogenicity [11]. For the serology study participants were categorised as colonized if *S. pyogenes* was detected by qPCR at any time point. Participants in the acquisition study group (negative at day 0) were categorised as newly-colonized if *S. pyogenes* was detected at either D7 or D21. All available serum collected on D21 from colonized participants was tested, along with a random selection from non-colonized participants (a 1:1.5 ratio of colonized to non-colonized participants). Of newly-colonized participants, a subgroup of 18 had paired serum (pre/post colonization) available for testing.

Enzyme-linked immunosorbent assay (ELISA) optimization was performed to determine optimal *S. pyogenes* protein coating concentrations, blocking buffer solution, serum dilution and secondary antibody concentration. 96-well flat-bottomed high binding ELISA plates were coated overnight with several recombinant proteins in pH 9.6 carbonate buffer: *S. pyogenes* cell envelope protease (SpyCEP) at 0.16µg/ml, *S. pyogenes* Adhesion and Division protein (SpyAD) at 1µg/ml, M1 protein at 0.5µg/ml, Mac/IdeS protein (henceforth referred to as Mac) at 1 µg/ml, and Collagen binding protein (Cpa) at 0.16 µg/ml. Proteins were provided by National Institute for Biological Standards and Control, MHRA, UK and derived from the reference SF370 strain(M1/*emm*1/FCT-2) (Supplementary data).[14] Blocking and serum dilution was performed using 1% casein blocking buffer (ThermoFisher Scientific, USA). Serum was diluted to 1:100 for Cpa and Mac, and 1:800 for M1, SpyCEP and SpyAD, and applied to protein coated wells. Bound IgG was detected with goat anti-human IgG-HRP conjugate (Invitrogen, USA), diluted to 1:500, then incubated with SureBlue TMB 1-Component Peroxidase Substrate (KPL). The reaction was stopped with 1% hydrochloric acid and optical density (OD) was read at 450nm, subtracting background OD. Six washes were performed between each ELISA step with 0.05% Tween-20 in phosphate buffered saline (PBS). In order to quantify anti-protein IgG activity in participant sera, 12 serial three-fold dilutions of pooled human immunoglobulin (Gammanorm, Octapharm) were used to generate standard curves commencing at 1:33.3 dilution (14.85mg/mL) in 1% casein buffer. Seroconversion between D0 and D21 was considered as a 2-fold rise in antibody titre, or 4-fold rise in antibody titre if raw optical density measurements were <0.25 at baseline, as per previously published definitions [15]

In order to measure antibody titres to the additional vaccine antigens Group A carbohydrate (GAC) and Streptolysin O (SLO), an optimized 4-plex (GAC, SLO, SpyCEP, SpyAD) serology

assay using the Luminex platform was performed on all available sera using protocol described [16]. All antigens were supplied by GSK Vaccine Institute for Global Health (GVGH). All sera with ELISA data were tested, except two samples from non-colonized participants, where there was no remaining volume. Samples were tested in duplicate at a dilution of 1:8100 alongside standard curves derived from pooled intravenous immunoglobulin (Privigen, CSL Behring). Mean florescence intensity was measured for each antigen with a Luminex 200 instrument (Invitrogen).

Statistical analysis: All statistical analysis was performed in R (version 4.0.1). Comparison of *S. pyogenes* colonization status and incident acquisition between children who received LAIV and those who did not was performed using chi-squared test. In addition, logistic and generalized mixed effects logistic regression models, accounting for multiple timepoint sampling from individuals, were used to explore the change in colonization status over time within the vaccinated and unvaccinated groups, as previously described [10]. Co-variates included were age in months, sex, the presence of asymptomatic respiratory viruses at baseline, timepoint (D0 vs D7, D0 vs D21) and receipt of LAIV [10, 11].

Samples where optical density measured by ELISA fell below the detection limit of the standard curve, were allocated a random value between zero and the limit of detection. Antibody titres in Luminex data were interpolated using xPonent 4.2 software (Luminex Corporation) using 5-parameter logistic regression. For the 4-plex Luminex assay, where both limit of accurate quantification and detection were characterized, results falling below respective limits were randomly assigned a value between zero and the lowest value of each limit [16]. Antibody data (IVIG–adjusted anti-protein activity in mg/mL for ELISA and Relative Luminex Units (RLU/mL) for Luminex assay) were Log transformed and assessed for normality using QQplot and the shapiro-wilk test. Log transformed antibody quantities between groups were compared using Student's t-test. Paired Student's t-test was performed to compare antibody levels before and after *S. pyogenes* colonization from individual participants. A p value of <0.05 was considered statistically significant. Correlation between Log transformed antibody titres to different antigens within individuals was assessed with Spearman's method.

RESULTS

S. pyogenes colonization prevalence and incidence: A total of 320 participants were included in this study, of which 212 received LAIV (Figure 1). Overall, 71/320 (22%) children were colonized with *S. pyogenes* on at least one time point within the 21-day study period. At D0 26/320 (8%) participants were colonized, 13 in the LAIV (6%) and 13 in control (12%) groups (p=0.068, Table S1). 109/320 (34%) participants had a baseline respiratory virus detected at D0. There was no difference in colonization at baseline between participants with a detectable respiratory virus and those without (11/109, 10% vs 15/211, 7.1%, p=0.4). In the acquisition study group, 45 (15%) had acquired colonization by D7 or D21 (Table 1), 35/199 (18%) in LAIV

group and 10/95 (11%) in control group (Table 1, p=0.12). A logistic regression model was used to explore the odds of new *S. pyogenes* colonization at either D7 or D21 in the acquisition study group (n=294), accounting for age, sex, LAIV receipt and the presence of other respiratory viruses at D0 (Table S2), showing no significant association between LAIV and *S. pyogenes* acquisition (OR 1.92, 0.93-4.31, p=0.09).

In order to explore changes in colonization status within an individual over time in all children (n=320), a generalized logistic mixed effects regression model was used to explore the odds of *S. pyogenes* colonization over time, accounting for age, sex, LAIV receipt and the presence of other respiratory viruses at D0 (Table S3). This model demonstrated a significant interaction between LAIV receipt and the D21 timepoint terms. Therefore we constructed separate models for the LAIV and control groups. The odds ratio (OR) of *S. pyogenes* colonization was higher at D21 compared to day D0 in the LAIV group (OR 3.18, 95% CI 1.49-6.81, p=0.003, Table 2) but not day 7 (OR 1.30, 95% CI 0.57-2.97, p=0.54). The OR of *S. pyogenes* colonization in the control group was not higher at either D7 (OR 0.34, 95% CI 0.09-1.27, p=0.11) or D21 (OR 0.86, 95% CI 0.3-2.51, p=0.79) when compared to D0 (Table S4).

Only 17 of 71 (23.9%) participants with *S. pyogenes* detected during the study were colonized at two timepoints, with only 2 (2.8%) colonized at all three study time points (Figure S1). No difference in qPCR quantified *S. pyogenes* density was observed between LAIV and control participants at any timepoint, nor in episodes with persistent positivity at subsequent study visits (Tables S5A and S5B).

We compared symptom data during the study period for all 294 children in the acquisition study group. Ten children had infected skin sores during the 21-day follow up, 9 in the LAIV group and 1 in the control group (5% vs 1%, p=0.2, Table S6). Only three episodes of sore throat were reported, all in the LAIV group (Table S6). Skin sores were more common in those who became colonized (9% vs 2% p=0.05) (Table S6). No statistically significant difference in fever, cough, rhinorrhoea, or sore throat was seen in children who acquired *S. pyogenes* compared to those who did not (Table S6). In the total study group only 6/71 (9%) colonized participants had either a sore throat or an infected skin sore.

Serological responses to *S. pyogenes* antigens: ELISA-quantified serum antibody titres at D21 in 40/48 colonized participants were compared with those in 61 randomly selected non-colonized participants (all from the LAIV group due to sera availability). Missing serum was due to insufficient remaining material from the parent study. The age was not significantly different between participants included in serological study who were colonized compared to non-colonized controls (median age in months 36 vs 32, p=0.3, Table S6). Sera from colonized participants demonstrated significantly higher IVIG-adjusted IgG levels to M1, SpyCEP, SpyAD and Mac, but not Cpa, compared to non-colonized participants (Figure 2A). Within individuals the strongest correlation was observed between IgG titres for M1 and for SpyCEP (Figure 2B). Paired serum was available for 18/35 newly-colonized participants. Mean M1- and SpyCEP-

specific IgG titres were significantly increased at D21 compared to D0, but not IgG titers to SpyAD, Mac or Cpa (Figure 2C). The proportion of newly-colonized participants who seroconverted pre/post colonization was greatest for M1 and SpyCEP (Figure 2C). To explore serological responses further, a recently described 4-plex assay was used to quantify antibodies to additional *S. pyogenes* vaccine antigens GAC and SLO, along with SpyCEP and SpyAD. This assay was tested on 99/101 samples with ELISA-quantified anti-*S. pyogenes* titres [16]. Sera from colonized children demonstrated significantly higher titres to GAC, SLO and SpyCEP but not to SpyAD (Figure 3A). Within individuals the strongest correlation was observed between IgG titres for SLO and for SpyCEP (Figure 3B). In the subset of participants with *S. pyogenes* acquisition during the 21-day study period (n=18), differences in IgG titers at D0 and D21 were not statistically significant (Figure 3C). Antibody titres to SpyCEP and SpyAD that were measured by both techniques were well correlated (Figure S2). Removing the participants with sore throat or infected skin sores and colonization during the study had no significant impact on the serological results, nor did excluding participants colonized on D21 only, nor applying a more conservative definition of seroconversion (4-fold increase in IgG titre regardless of baseline optical density) (Figures S3, S4, S5).

DISCUSSION

In this post-hoc study of a randomized controlled trial in The Gambia, we analysed the impact of LAIV on *S. pyogenes* nasopharyngeal colonization in children aged 24-59 months. Using PCR to define colonization events, we observed an 8% prevalence at study baseline. Our data demonstrate only a modest impact of LAIV on colonization rates. We observed non-significantly increased rates and odds of new colonization in the LAIV group and increased odds of colonization at D21 compared to baseline in the LAIV group only. During the 21-day study, 22% of children were colonized at one or more timepoints, with most (73%) only colonized once. The dynamic picture of colonization we observed may be partially influenced by LAIV administration and reflects the common exposure of children to *S. pyogene*s in areas with a high burden of disease. Our findings are consistent with published estimates from LMICs [4, 17].

S. pyogenes was responsible for substantial mortality in influenza pandemics of 1918 and 2009 [8, 9]. The impact of respiratory viruses on pharyngeal colonisation, modulation of host-pathogen interaction and promoting severe *S. pyogenes* disease is complex and poorly understood[8, 9, 18-21]. While mice administered a non-lethal challenge with *S. pyogenes* following an influenza challenge frequently had a severe and fatal disease course [21], mice vaccinated with LAIV were protected from *S. pyogenes* superinfection [20]. Few models have specifically investigated the impact of respiratory viruses on pharyngeal colonization [22]. Through the *S. pyogenes* human challenge model, co-challenge studies with LAIV could reveal mechanistic insights into the interaction between influenza virus, *S. pyogenes*, and host immune responses [23]. A co-challenge study with *S. pneumoniae* and LAIV has shown how virus-

induced inflammatory responses and impaired innate immune responses promote bacterial colonization [24]. Whilst no impact on colonization density was demonstrated in this study, it is possible that the observed rates of acquisition in the LAIV group was due to modestly increased colonization density, reaching a threshold for PCR positivity. This mechanism has been observed for other bacteria including *S pneumoniae* both in this study population and in other settings.[10, 25] Our data provide further evidence that the impact of LAIV on colonization with potentially pathogenic bacteria is only modest and supports the wider rollout of LAIV to reduce influenza disease and its complications in LMICs.

We also demonstrate that asymptomatic *S. pyogenes* colonization leads to seroconversion to several antigens representative of different stages of *S. pyogenes* infection [14], with higher responses observed in colonized children compared to non-colonized controls. Given that prior exposure to *S. pyogenes* in all children in this cohort would be similar, these differences likely reflect recent exposure. The most notable responses were seen to full-length streptococcal M1 protein and to the envelope protease SpyCEP, Reactivity to full length M1 protein likely reflects activity to conserved M protein region rather than type-specific reactivity, given that *emm*1 isolates have not been identified in The Gambia[26, 27] IgG antibodies to other antigens, SpyAD, Mac, GAC and SLO, were higher in children where *S. pyogenes* colonization had been detected, but not Cpa, The Cpa used in our study was originally derived from the M1 FCT-2 strain SF370. Our previous study found no Gambian FCT-2 isolates, although approximately 70% were Cpa-positive FCT-3 or FCT-4 isolates[27]. However, Cpa from FCT-3 or FCT-4 shares only ~50% amino acid identity with Cpa from FCT-2 which may explain limited Cpa reactivity in this cohort. Noting that the antigens tested via ELISA and Luminex were obtained from different sources, there was broad concurrence between the two serological assays, except that IgG to SpyAD was significantly higher in colonized compared to non-colonized participants when measured by ELISA and not by Luminex. A detailed comparison of the two platforms to determine the optimal technique for measurement of IgG to these *S pyogenes* antigens was not performed. Nonetheless, our findings suggest that asymptomatic pharyngeal colonization may induce an IgG immunological response to multiple antigens.

The definition of "true" *S. pyogenes* colonization, refering to colonization *without* a serological response, risks oversimplification of a complex and dynamic state influenced by host-immunity, bacterial characteristics, and environmental factors. This serologically inactive phenotype has been described with minimal serum antibody response to SLO and anti-DNAase B (7, 32). Our data shows that serological responses following asymptomatic colonization vary accross different proteins. Furthermore, in practice colonization is often defined as an asymptomatic person with detectable *S. pyogenes* without serial serological testing. In detailed longitudinal analyses, seroconversion has been documented following asymptomatic acquisition of *S. pyogenes* in the USA and Egypt, with highest proportion of seroconversions to type-specific M peptides and SpyAD [15, 28]. In another cohort study in the USA, seroconversion to type-

specific M protein frequently occurred following asymptomatic acquisition and conferred protection from homologous strain reinfection [29].

Protection following asymptomatic colonization and *S. pyogenes* intranasal vaccination has been observed, but the responsible mechanisms and serological corelates of protection have not been identified [30-35]. Early prospective observation demonstrated the emergence of acute rheumatic fever following asymptomatic *S. pyogenes* infection [36]. Recent evidence suggests that patients with rheumatic fever have more serological activity to S. pyogenes M peptides and conserved antigens than matched controls, including after asymptomatic pharyngeal colonization[37, 38]. Asymptomatic colonization may therefore contribute to pathological immunity in endemic settings and warrants further exploration.

Our study has several key limitations. Firstly, it was a post-hoc analysis of a study that was not designed or powered to assess the impact of LAIV on *S. pyogenes* colonisation. LAIV was used as a proxy for natural influenza infection and the observations may not reflect the true dynamics of *S. pyogenes* colonization during natural influenza infection. We defined colonization events by PCR and not by gold-standard microbiological culture of *S. pyogenes*. Whilst *S pyogenes* skin infection in children is common in this setting[39], we did not perform microbiological culture on children reporting infected skin lesions nor sore throats in this study. Nonetheless, exclusion of all participants with either a sore throat or infected skin lesion did not dramatically alter the serological findings. Serum IgG could only be measured in the LAIV group, so no serological comparison between vaccinated and unvaccinated groups was possible. Finally, PCR to a single preserved and specific *S. pyogenes* target does not allow for assessment of *emm*-type specific immunity, which is an historically important consideration for *S. pyogenes* serological activity.

Nonetheless, our study provides several important findings. Understanding both naturally occurring protective immunity and pathological autoimmunity to *S. pyogenes* from settings with the highest disease burden is of paramount importance for progress towards a safe and effective *S. pyogenes* vaccine. Our study adds further evidence that asymptomatic colonization may be immunologically significant, particularly in the context of influenza co-infection. Further research combining longitudinal observation, with detailed microbiological and immunological investigation should be prioritised from areas of high disease prevalence to gain a deeper understanding of immunity to this major human pathogen.

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FOOTNOTE PAGE

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Conflicts of interest. O.R. and M.C are employees of the GSK group of companies. GSK Vaccines Institute for Global Health Srl is an affiliate of GlaxoSmithKline Biologicals SA. OR reports ownership of GSK share options. We declare there are no conflicts of interest among other authors

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Figure 1. Study profile. 320 participants randomized 2:1 to the LAIV (day 0 vaccine) or control (day 21 vaccine) group. All participants (colonization study group) had S. pyogenes colonization status determined by real-time PCR at day 0, 7 and 21. 294 participants (the acquisition study group) were negative at baseline. Only participants receiving LAIV at day 0 had serum taken at days 0 and 21 (n=212). Serum from day 21 was available from 40/48 participants from LAIV group who were colonized at any time point and from 61/164 randomly selected non-colonized participants. Paired serum (pre/post colonization) was available from 18/35 participants who acquired colonization during the study period. LAIV = Live attenuated Influenza Vaccine. Created with BioRender.com

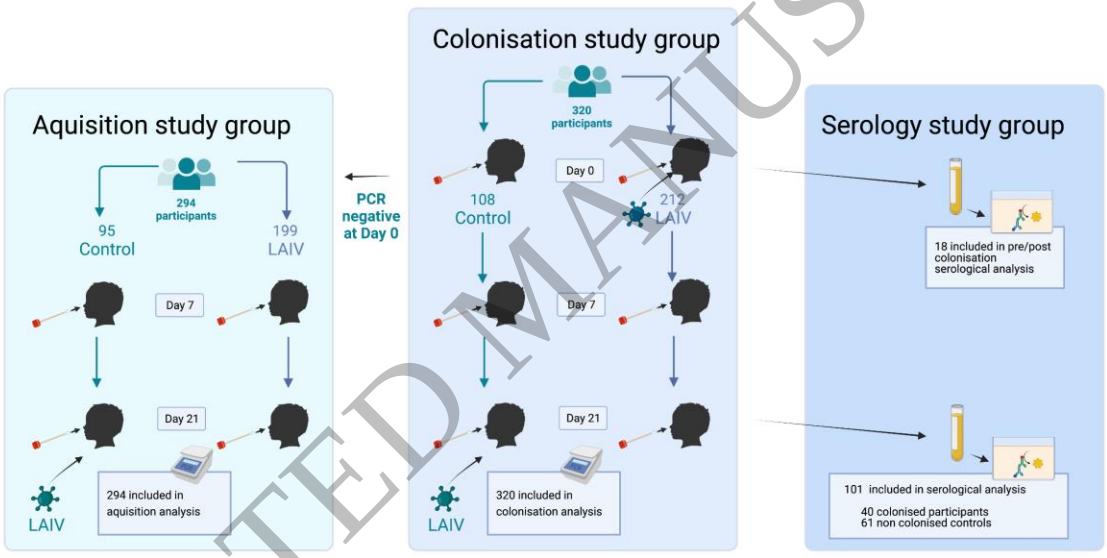

Figure 2. Serological responses to S. pyogenes colonization measured by ELISA. A. Comparison of anti-protein IgG activity to Cpa, M1, Mac, SpyCEP, and SpyAD in participants (n=101) according to anytime S. pyogenes colonization status. **B.** Pairwise correlation coefficients (Spearman's method) of IgG titre measured by ELISA within individual participants (n=101) **C**. Paired comparison of anti-protein IgG activity to Cpa, M1, Mac, SpyCEP, and SpyAD between day 0 and day 21 in newly colonized participants (n=18). Log_{10} transformed IVIG-adjusted anti-protein activity was compared with t-tests (unpaired and paired respectively), horizontal line depicts the median value. **D**. Percentage of study participants (n=18) acquiring S. pyogenes during the study who seroconverted between day 0 and day 21.

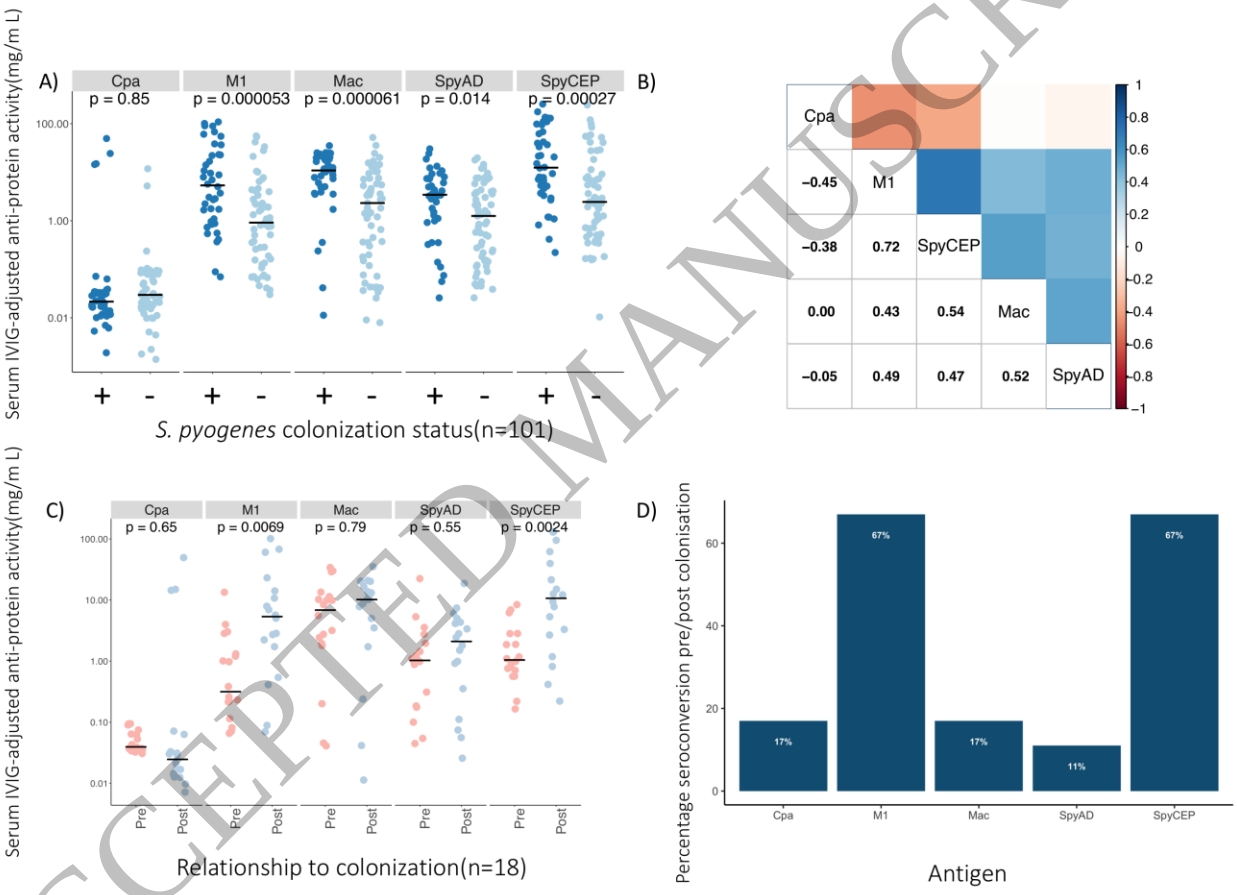

Figure 3. Serological responses to S. pyogenes colonization measured by Luminex 4-plex. **A.** Comparison of IgG activity to GAC, SLO, SpyCEP, and SpyAD in participants (n=99) according to anytime S. pyogenes colonization status. **B.** Pairwise correlation coefficients (Spearman's method) of IgG titre measured by Luminex within individual participants (n=99) **C.** Paired comparison of IgG activity to GAC, SLO, SpyCEP, and SpyAD between D0 and D21 in newly-colonized participants (n=18). Log_{10} transformed IVIG-adjusted anti-antigen activity was compared with t-tests (unpaired and paired respectively). Horizontal bar depicts the median value.

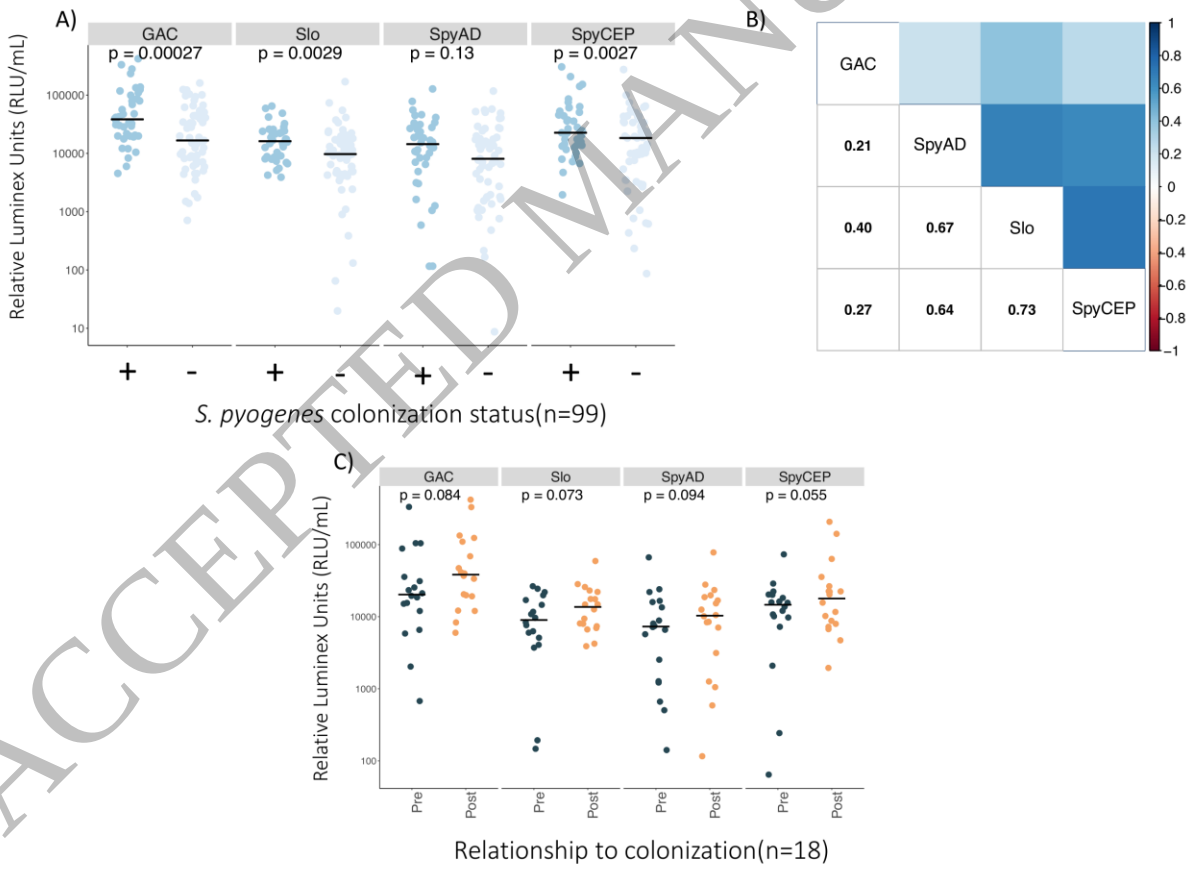

Table 1. Streptococcus pyogenes acquisition during the 21-day study period. Included below are n=294 in the acquisition study group.

Time point	*N*	*Overall, N = 294*[1]	*Control, N = 95*[1]	*LAIV, N = 199*[1]	*p-value*[2]
Day 7	294	18 (6%)	3 (3%)	15 (8%)	0.14
Day 21	294	35 (12%)	8 (8%)	27 (14%)	0.2
Any time	294	45 (15%)	10 (11%)	35 (18%)	0.12

[1]n(%)
[2]Pearson's Chi-squared test

Table 2. Factors associated with S. pyogenes colonization in the live attenuated influenza vaccine (LAIV) group (n=212).

	OR	*95% CI*	*p-value*
Day 7 (vs day 0)	1.30	0.57-2.97	0.540
Day 21 (vs day 0)	3.18	1.49-6.81	**0.003**
Positive respiratory virus at day 0	1.54	0.72-3.29	0.266
Age in months	0.97	0.93-1.01	0.162
Sex Male (vs female)	0.97	0.47-2.04	0.945

p-values for factors associated with Streptococcus pyogenes colonization are derived from a generalized logistic mixed effects model, taking into account changes within individuals over time. OR = odds ratio, CI = confidence interval.