Title page

Controlled human infection in late pregnancy: a single-arm interventional pilot trial investigating mother-to-infant transmission of *Neisseria lactamica*

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Abstract (300 words)

Background

The infant respiratory microbiome is derived largely from its mother and is associated with downstream health and disease. Manipulating maternal respiratory flora peripartum to influence the infant microbiome has not previously been investigated. *Neisseria lactamica* (Nlac) is a harmless pharyngeal commensal that correlates inversely with *N. meningitidis* carriage and disease. Intranasal Nlac inoculation is a safe and well-characterised controlled human infection model (CHIM) in non-pregnant healthy adults. We hypothesised that Nlac inoculation in pregnancy induces mother-to-infant Nlac transmission postnatally.

Methods

Twenty-one healthy pregnant women were inoculated at 36-38 weeks' gestation with 10^5 colony-forming units Nlac Y92-1009 at University Hospital Southampton Clinical Research Facility, UK (NCT04784845, closed to participants). Nlac selective culture, genome sequencing and serology were performed on maternal and infant oral, nasopharyngeal, breastmilk and serum samples over 15 weeks postpartum. Seven women naturally-colonised with Nlac at baseline were followed-up, but not inoculated. Oral samples were obtained from 12 one- to five-year-old siblings. The primary endpoint was infant Nlac colonisation.

Findings

Although 15/21 (71%) inoculated women became Nlac-colonised, no sustained Nlac Y92-1009 transmission to their infants was observed. Conversely, non-Y92-1009 Nlac strain-sharing was observed in 4/7 (57%) uninoculated mother-sibling pairs, and *M. catarrhalis* (Mcat) strain-sharing in 9/24 (38%) mother-infant pairs completing the study. Anti-Nlac serum IgG titres increased in 7/8 (88%) Nlac Y92-1009-colonised women, but none of their infants (where paired sera were available). There were no serious adverse reactions to the inoculum.

Interpretation

This first-in-human trial demonstrates that CHIM in pregnancy is feasible, and that Nlac Y92-1009 can safely and efficiently colonise pregnant women. Lack of sustained mother-to-infant Nlac transmission, despite evidence supporting mother-to-infant Mcat and sibling-to-mother Nlac transmission, challenges conventional perceptions of infants as passive recipients of maternal microbes, suggesting that respiratory commensal transmission is selective and microbe-specific.

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Research in Context

Evidence before this study

The infant upper respiratory tract (URT) microbiome is seeded by multiple maternal niches, including maternal URT, skin, breastmilk, vagina, and gut, and is also influenced by presence of co-habiting siblings. Recent advances in microbiome research have increasingly focussed on strain-level (rather than species- or genus-level) community profiling, demonstrating that a subset of maternal strains disproportionately shape the infant URT. Indeed, shared strains account for 95% of infant but only 7% of maternal oral microbes. The composition and developmental trajectory of the infant URT microbiome is associated with downstream health and disease, including recurrent URT infections and asthma. The causal mechanisms (if any) underlying these findings remain unclear, although may be partly mediated by competition between URT healthy commensals and bacterial and viral potential pathogens. Although such competitive relationships have been characterised using animal and in vitro models, infant URT commensalisation, mother-to-infant URT microbiome transmission, and the role of the infant URT microbiome in downstream health and disease have not hitherto been investigated in interventional trials. We searched Pubmed from inception to August 25th 2024, with no language restriction, using the terms ("microbiome" OR "microbiota") AND ("respiratory" OR "pharyngeal" OR "pharynx" OR "oral" OR "mouth" OR "nasal" OR "nose") AND ("mother" OR "maternal") AND ("infant" OR "newborn") AND "transmission" AND "strain". Of the 12 results, four were observational studies describing strain-level mother-to-infant URT microbiome transmission, and one was the published protocol for the study reported in this paper.

Additionally, we searched Pubmed from inception to August 25th 2024, with no language restriction, using the terms "Neisseria lactamica" AND "Neisseria meningitidis" AND ("colonisation" OR "carriage"). This search yielded 55 results, of which 11 were observational studies describing the inverse epidemiological relationship between pharyngeal *Neisseria lactamica* (Nlac) and *N. meningitidis* (Nmen) colonisation, and three were interventional controlled human infection model (CHIM) studies. The latter three studies reported safety data from over 400 healthy non-pregnant adults, and demonstrated that Nlac inoculation directly reduces pharyngeal Nmen carriage in a serogroup-independent manner. The mechanisms

underlying this effect are unclear but may involve competition for niche occupation, with evidence supporting induction of cross-reactive adaptive immunity.

Added value of this study

This study is, to our knowledge, the first ever perinatal respiratory CHIM, demonstrating this approach can be ethically acceptable and practically feasible. Within the limits of a proof-of-concept study, the inoculation strain was safe and efficient at inducing maternal URT Nlac colonisation, although this appears insufficient to induce infant Nlac colonisation. This study also challenges past evidence reporting that Nlac carriage is uncommon in adults (10% of women over 20 years old). Indeed, baseline carriage of naturally-acquired Nlac strains was 41% (7/17) in uninoculated pregnant participants with one- to five-year-old children (compared to 0/14 in those without), on par with the epidemiological colonisation peak seen in young children themselves. From this, we infer that Nlac is readily transmitted from children to their mothers, despite apparent lack of mother-to-infant transmission.

Implications of all the available evidence

Our finding that inoculation-induced maternal Nlac colonisation is insufficient to cause mother-to-infant transmission, despite evidence of mother-to-infant *Moraxella catarrhalis* (Mcat) transmission, suggests that infant commensalisation is a selective process. These results have implications for future CHIM trials, which may involve health-associated commensals already known to transmit naturally from mothers to infants.

Introduction

The infant upper respiratory tract (URT) is often colonised asymptomatically by pathobionts (potential pathogens) that can precipitate pneumonia, meningitis and asthma, each of which are common causes of morbidity and mortality in children aged under five.[1] Pathobiont acquisition and symptomatic infection is influenced by the composition and developmental trajectory of the infant URT microbiome.[2] Disruption of the stable microbiome is associated with mucosal inflammation and invasion by pathobionts and viruses,[3] although interventional human studies providing causal evidence of this are limited.[4] The infant URT microbiome is largely derived from maternal microbes, with seeding from maternal URT, breastmilk, skin, gut, and vagina.[5]

The possibility of favourably manipulating the maternal URT microbiome (and that of the infant by horizontal mother-to-infant transmission) has been raised, but not hitherto investigated.[4] An experimental model demonstrating that mother-to-infant URT microbiome transmission can be manipulated would then pave the way for testing health-associated URT commensals of infancy (such as *Dolosigranulum* and *Corynebacterium* species) or early childhood (such as *Neisseria lactamica*).

N. lactamica (Nlac) is an acapsulate pharyngeal commensal that colonises over 40% of one- and two-year-old children,[6] but is rarely isolated from the throats of adults and neonates.[7] Epidemiologically, Nlac carriage correlates inversely with *N. meningitidis* (Nmen) colonisation[8] and invasive meningococcal disease (IMD),[7] with mathematical modelling suggesting that protection conferred by Nlac persists over five years.[9] A controlled human infection model (CHIM) of Nlac URT carriage is safe,[10,11] and the inoculation strain Y92-1009 is genetically stable over 26 weeks of colonisation.[12] Inoculation-induced Nlac carriage reduces Nmen colonisation (independent of serogroup) from 18% (11/61) to 8% (5/61),[13] an effect that persists for at least 26 weeks and is associated with expansion of cross-reactive B lymphocytes.[14]

We conducted a CHIM trial with Nlac strain Y92-1009 to test the hypothesis that nasal inoculation in late pregnancy induces mother-to-infant Nlac transmission with neonatal colonisation by fifteen weeks postnatally. Based on results of previous CHIM trials,[15] we predicted that mothers would become colonised with Nlac, but did not know whether Nlac

would be transmitted to the infant. We anticipated that anti-Nlac and anti-Nmen serum IgG titres would increase in Nlac-colonised, but not uncolonised, women and infants. As Nlac colonisation is uncommon in adults and infants, whereas *Moraxella catarrhalis* (Mcat) is readily transmissible from mother to infant and is present in a third to 100% of infants older than one month,[16] we hypothesised additionally that naturally-occurring mother-to-infant Mcat transmission would be observed using the same culture methods, regardless of whether inoculation-induced infant Nlac Y92-1009 colonisation was seen.

Nlac Y92-1009 was felt to be a suitable agent for investigating safety and efficacy of CHIM in pregnancy, as it is known to be safe and effective in non-pregnant adults. Demonstration of safety in pregnant women was also felt to be a necessary prerequisite before direct neonatal CHIM could be considered. Additionally, as the core aim was to investigate mother-to-infant commensal transmission, the choice of a commensal that is uncommon in infants and adult women was desirable, to probe experimentally-induced (as distinct from naturally-occurring) transmission. Although Nlac appears to protect against Nmen carriage, this proof-of-concept study did not aim to establish whether infant anti-meningococcal responses could be induced by maternal Nlac inoculation.

Methods

Study design and participants

In this single-arm CHIM study, healthy pregnant women were inoculated intranasally with 10^5 colony-forming units (CFU) Nlac. Participants were screened and enrolled at 34-37 weeks' gestation (Visit 1) and inoculated at 36-38 weeks' gestation (Visit 2), after which mother-infant pairs were followed-up at 0-24 hours (Visit 3), 7±3 days (Visit 4), 28±3 days (Visit 5) and 15±2 weeks (Visit 6) postpartum (Figure 1A). Telephone pre-screening was conducted to identify potentially eligible participants, followed by screening and inoculation visits at University Hospital Southampton NIHR Clinical Research Facility; most birth visits took place at the Princess Alexandra Hospital Southampton and most follow-up visits at the participants' own homes. Adult women with a healthy singleton pregnancy were eligible, while those with immunosuppression or recent or planned use of antibiotics were excluded (full inclusion and

exclusion criteria and sample size rationale in Appendix 1 p2). Written informed consent was obtained from all participants at the point of enrolment.

The trial was approved by the London Central Research Ethics Committee (21/PR/0373), sponsored by the University of Southampton (ERGO 61640), registered on ClinicalTrials.gov (NCT04784845), and overseen by an independent safety committee. Additional study design details are in the abbreviated published protocol,[10] and in the full study protocol (https://eprints.soton.ac.uk/492361/).

Procedures

Participant sampling

Upper respiratory swabs were obtained (maternal oropharyngeal, nasopharyngeal and saliva at Visits 1-6; infant nasopharyngeal and saliva at Visits 3-6), as well as optional breastmilk (hand-expressed by participants into sterile specimen containers; Visits 3-6) and blood samples (umbilical cord at Visit 3; infant venous at Visits 5 and 6; maternal venous at Visit 6).

Optional anonymous questionnaires (Visits 1 and 5) were used to investigate participant motivations, concerns and perceptions, the results of which have been published.[17] Participants were compensated up to £100 for their involvement in the study. Adverse events and clinical data, including mode of delivery and infant feeding, and antimicrobial use, were recorded throughout the study period. Household COVID19 infection (defined as a positive lateral flow or laboratory test in any household member) was recorded, but no SARS-CoV2 testing was performed as part of the study. Participants already colonised with Nlac (non-Y92-1009) at baseline were not inoculated, but were followed up as for inoculated participants.

Inoculum preparation

A dedicated batch of lyophilised (freeze-dried) Nlac (hereafter LyoNlac) was produced and stored under Good Manufacturing Practice (GMP)-like conditions at the University of Southampton (Appendix 2, p3), using frozen seed stocks of Nlac Y92-1009 (sequence type 3493, clonal complex 613) produced by the GMP facilities at UK Health Security Agency (Porton Down, Salisbury).[14] Immediately prior to use, each ampoule of LyoNlac was reconstituted in sterile phosphate-buffered saline (PBS; Fisher Scientific), serially diluted to 10^5 CFU/mL, and

delivered nasally using a sterile pipette. Inoculum purity and viability were confirmed during batch production, and again for each ampoule at the point of inoculation.

Selective culture

Each flocked URT swab (Medical Wire) was transported in 1mL storage medium comprising 10% glycerol in 0·1% diethylpyrocarbonate (DEPC)-treated water (Invitrogen). After vortexing participant samples for 30 seconds to produce suspensions, 200μL of each was spread over GC agar supplemented with 40μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated at 37°C, 5% CO_2. Nlac growth was confirmed by oxidase test, Gram stain, microscopic morphology analysis and either matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) or analytical profile index, and colonisation density was calculated (CFU/mL).[14] These methods were also used to identify Mcat throughout the study, which is readily culturable on GC agar.[18] Residual respiratory sample suspensions, breastmilk and pure growth isolates (up to five for Nlac and one for Mcat per sample) were appropriately prepared and cryopreserved at-80°C.

Whole genome sequencing

DNA extraction and whole genome sequencing (WGS) were performed by MicrobesNG (Birmingham, UK)[19] on up to three Nlac isolates per sample for the first and final two samples that were culture-positive for each participant. Additionally, for any families where at least two individuals were colonised with Mcat, up to three isolates were sequenced per individual. Appendix 3 (p4) lists version numbers and digital online identifiers for all software and databases used in analysis.

In brief, DNA extracts were used to prepare amplicon libraries (Nextera XT Library Prep Kit, Illumina; Microlab STAR automated liquid handling system, Hamilton), and sequencing was performed on an Illumina NovaSeq 6000 (Illumina). Resulting reads were trimmed (Trimmomatic) and assembled (SPAdes). For uninoculated participants naturally-colonised with non-Y92-1009 Nlac strains at baseline, a single isolate from each participant underwent additional long-read sequencing, with libraries prepared using Oxford Nanopore Technologies (ONT) SQK-LSK109 kit with Native Barcoding EXP-NBD104/114, and sequencing on a GridION with FLO-MIN106 R·9·4·1 flow cell (ONT). Hybrid assembly was performed (Unicycler) and all

short-read and hybrid assemblies were annotated (Prokka), followed by quality assessment (QUAST). The 16S rRNA gene sequence was used to report species identity, based on the SILVA database.

Assemblies returned by MicrobesNG were aligned using Snippy, with reference genomes Nlac Y92-1009 (BioProject accession PRJNA331097) and Mcat CCRI-195ME (PRJNA350869). Variant distance was computed for each genome pair, using bcftools to intersect pairs of variant call files and snp-dists to produce a single nucleotide polymorphism (SNP) distance matrix, with and without correction for putative recombinant sites using Gubbins. Genome pairs differing by fewer than ten recombination-corrected SNPs were classed as the same strain, compared with possible strain-sharing for ten to 100 SNPs, and distinct strains for 100 or more SNPs; consensus guidance regarding SNP distance thresholds is lacking, with most literature focussing on pathogens in outbreaks rather than sustained commensal colonisation.[20] Phylogenetic trees were generated with RAxML-NG, specifying a general time reversible model with gamma-distributed rate heterogeneity set to ten parsimony and ten random starting trees, using maximum likelihood, Felsenstein bootstrapping (100 replicates), and optimisation for near-zero branch lengths. Variant distance matrices were visualised using pheatmap in RStudio, and phylogenetic trees were visualised online using the Interactive Tree of Life.

Enzyme-linked immunosorbent assays

Nlac- and Nmen-derived deoxycholate-extracted outer membrane vesicles (Nlac-dOMV and Nmen-dOMV, respectively) were prepared as previously described,[21] and used to perform serum immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISA).[22] Briefly, 96-well plates were pre-coated with 50μL of one of: 20μg/mL Nlac-dOMV, 20μg/mL Nmen-dOMV or 20μg/mL bovine serum albumin (BSA, negative control) in carbonate coating buffer (pH 9·6). All coated wells were blocked with 200μL of 5% foetal calf serum in PBS (PBS-FCS). Each serum sample was serially diluted two-fold in sterile PBS, and, after washing blocked wells, duplicate 50μL aliquots of each serum dilution were loaded and incubated for 1h. After subsequent sequential incubations with (i) biotinylated rat-derived anti-human IgG (BioLegend) in PBS-FCS (1:1,000), and (ii) streptavidin horseradish peroxidase (Abcam) in PBS-FCS (1:1,000), 100 μL of the chromogenic substrate, *o*-phenylenediamine dihydrochloride (Fisher Scientific), was added to each well. Colour change was measured as optical density at 490nm (OD_{490nm}) using an iMark

microplate reader (Bio-Rad). Single-donor positive control serum (with high IgG titre against both Nlac-dOMV and Nmen-dOMV) was available from a previous Nlac CHIM.[19] A four-parameter logistic log curve of reciprocal dilution against OD_{490nm} was fitted to titrations of the positive control serum for each plate, and used to interpolate reciprocal dilution for measured OD_{490nm} of each sample dilution, expressed as a percentage of the positive control titre (Appendix 4, p5).

Visit 6 amendment

Collection of maternal and infant venous blood at Visit 6 was incorporated by study amendment (approved 8th March 2022), based on pertinent findings in a related Nlac CHIM study.[15] As the amendment was approved after study recruitment had completed, pre-inoculation maternal venous blood samples were not available, and umbilical cord blood was used as an imperfect proxy for maternal baseline serological status. The Visit 6 amendment also allowed for collection of saliva samples from co-habiting children aged under 5-years-old (hereafter 'siblings'). The rationale, implications and supplementary results relating to this amendment are detailed in Appendix 5 p6.

Outcomes

The primary outcome was confirmation of infant Nlac colonisation by selective culture of respiratory samples. Secondary and exploratory endpoints included: Nlac colonisation density in inoculated participants compared with their infants; comparison of Nlac genome sequences for isolates derived from inoculated and uninoculated participants and their infants; Mcat genome sequence for isolates derived from inoculated participants compared with their infants, to assess for evidence of natural commensal strain-sharing; association between Nlac colonisation and serum immunoglobulin G against Nlac outer membrane vesicles in inoculated participants and their infants; Nlac oral colonisation in co-habiting children under five years old; and assessment of participants' perspectives on research participation. Safety endpoints included the number of participants with adverse reactions to the inoculum, and any serious adverse events during the study period.

Statistical analyses

GraphPad Prism was used to perform data analyses. Quoted p-values are two-tailed and considered statistically significant if $\leq 0{\cdot}05$, and interquartile range (IQR) is quoted for all medians. Normality was assessed by the Shapiro-Wilk test. Mann-Whitney test was used to compare independent non-parametric data, and Wilcoxon matched-pairs signed rank test to compare median immunoglobulin titres across timepoints. Correlation between continuous non-parametric data was assessed by Spearman's rho (r_s). Fisher's test was used to investigate associations between categorical data. Sample size calculation and rationale is detailed in Appendix 1 (p2).

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

All visits took place between 1st October 2021 and 12th July 2022. Following telephone pre-screening of 39 individuals, 31 women were enrolled, although three were subsequently withdrawn prior to inoculation (Figure 1B). Seven women were already colonised with Nlac (non-Y92-1009) at screening, and the remaining 21 were inoculated, of whom seven (100%) and 17 (81%) completed all follow-up (Visits 3-6), respectively. For the 24 participants completing the study, the dataset was complete with no missing data, and saliva samples were obtained from all 12 siblings (Visit 6). Participant characteristics are shown in Table 1. As follow-up visits were timed relative to infant age, and as gestational age at birth ranged from 38 to 42 weeks, there was a degree of expected variation in visit timing relative to inoculation (Figure 1C). When surveyed anonymously, participants mostly reported altruistic motivations, such as contributing to science and helping other mothers and babies, while their concerns mainly related to their baby's potential discomfort due to blood and respiratory sampling.[17] Adverse events are detailed in Appendix 6 (p7), and there were no serious adverse reactions to the inoculum.

Maternal and infant respiratory samples were assessed for culturable Nlac (Figure 2) and Mcat, with strain-sharing investigated by isolate genome sequencing (details below; Figures 3 and 4). Nlac Y92-1009 was identified in 15/21 (71%) inoculated women, and in 13/17 (76%) women completing the study (Figure 2A). Conversely, Nlac Y92-1009 was only detected in one infant born to an inoculated woman, at a single timepoint (Visit 3, birth; saliva), with no evidence of sustained infant colonisation. The inoculation strain was not cultured from any siblings, although a naturally-acquired Nlac strain was identified in one sibling (saliva). We identified Mcat strain-sharing in 6/17 (35%) mother-infant pairs, suggesting Mcat transmission despite lack of Nlac Y92-1009 transmission (details below; Figure 4).

Exposure to antibiotics (prescribed for indications unrelated to the study) was associated with reduction in maternal Nlac colonisation density, loss of colonisation, and failure to establish colonisation (Figure 2A and 2B). Indeed, antibiotics were received by 8/17 (47%) inoculated women completing the study, and in 6/8 (75%) of these women, Nlac was not cultured on some or all sampling visits. Conversely, in all nine of the women who received no antibiotics, Nlac was cultured at least once, and at every post-inoculation visit in 6/9 (67%). Most antibiotic exposure occurred prior to Visit 3 (birth), particularly in the peripartum period, and the association between antibiotic use and colonisation density was strongest at this visit (median density 0 [IQR 0-15] CFU/mL in antibiotic-exposed participants compared with 9,320 [559-31,020] CFU/mL in unexposed participants, p=0·012; Figure 2B).

Maternal Nlac colonisation density was negatively correlated with elapsed time following inoculation (r_s -0·25, p=0·037 in 17 women completing the study; r_s -0·55, p=0·0005 in nine women who received no antibiotics; Figure 2C). Loss of Nlac colonisation occurred after COVID19 infection in two women (neither of whom received antibiotics), although there was no significant association between COVID19 infection and colonisation density (data not shown).

Nlac (non-Y92-1009) colonisation was detected at screening in 7/17 (41%) pregnant women with co-habiting one- to five-year-old children ('siblings'), compared with 0/14 women without (p=0·0087; Figure 2A). Nlac (non-Y92-1009) was identified at Visits 5 and 6 (11 weeks apart) in one infant born to an uninoculated woman, with mother-infant strain-sharing confirmed by genome sequencing. Nlac was first cultured from this infant 50 days after the mother's only

positive sample (Visit 1, screening), and 27 days after the mother's first negative sample (Visit 3, birth), while the infant's sibling was not colonised at Visit 6. It is not possible to confirm whether the infant acquired Nlac from its mother or sibling.

Median colonisation density was numerically lower in uninoculated compared with inoculated women, particularly at Visits 3 and 4 (median 6 [IQR 0-2,024] and 3 [0-23] CFU/swab compared with 724 [0-20,540] and 217 [0-4,700] CFU/swab, respectively), although these differences were not significant, even when accounting for antibiotic use (Figure 2D).

For the 70 maternal oropharyngeal samples that were culture-positive for Nlac (Y92-1009 and non-Y92-1009), 23/70 (33%) of corresponding maternal saliva samples were also culture-positive. Conversely, Nlac was not identified in any saliva samples where the corresponding maternal oropharyngeal sample was culture-negative. Maternal saliva and oropharyngeal samples were concordant for 104/151 (69%) study visits (both positive 23/104, both negative 81/104). Nlac was not cultured from any nasopharyngeal or breastmilk samples, and Nmen was not cultured from any sample type.

To investigate for household strain-sharing and longitudinal genomic stability, 183 Nlac isolates were analysed by WGS (Figure 3A). All Nlac Y92-1009 genomes were highly similar, with median recombination-corrected SNP count = 0, total SNP count = 1, and total variant count (including insertions, deletions, and translocations) = 26 (IQR 0-1, 1-3, and 23-30, respectively; Figure 3B). Variant count was positively correlated with elapsed time since inoculation (r_s 0·72 [p<0·0001], 0·50 [p<0·0001], and 0·32 [p=0·0010] for total variants, total SNPs, and recombination-corrected SNPs, respectively; Figure 3C).

For non-Y92-1009 Nlac strains, strain-sharing was confirmed for one mother-infant and two mother-sibling pairs, while in two mother-sibling pairs, individuals were colonised with distinct Nlac strains (Figure 3D). Non-Y92-1009 Nlac strains that were similar to each other (10-100 SNP distance) were detected in seemingly unrelated individuals from five families. Findings were confirmed by phylogenetic analysis (Figure 3E). Co-colonisation with more than one Nlac strain was not detected in any individual, although this cannot be ruled out, as only three colonies were sequenced per sample.

Evidence of naturally-occurring mother-infant commensal transmission was sought by sequencing 52 Mcat isolates derived from 14 families (Figure 4A). This suggested probable (0-10 SNP distance) within-family strain-sharing for seven mother-infant pairs, one mother-infant-sibling triad, and one infant-sibling pair, and possible (10-100 SNP distance) strain-sharing for one further mother-infant pair (Figure 4B). These findings support mother-to-infant Mcat transmission, despite lack of apparent mother-to-infant Nlac transmission. Probable or possible strain-sharing was also seen between individuals from ten unrelated families. This may represent exposure to common circulating strains, although a direct epidemiological relationship between these families cannot be ruled out; indeed, all ten families included siblings attending childcare with unrelated children.

As colonisation with Nlac Y92-1009 for longer than two weeks is known to cause an increase in anti-Nlac-dOMV and anti-Nmen-dOMV IgG titres in adults,[15] we hypothesised that increased anti-Nlac-dOMV and anti-Nmen-dOMV IgG titres would be seen in infants that acquired Nlac, compared with decreased transplacental maternal antibodies in uncolonised infants over time[23]. Paired sera from Visit 3 (umbilical cord) and Visit 5 (infant) blood samples were available for 7/24 (29%) of infants, demonstrating a significant decrease in IgG titres against Nlac-dOMV (median titre change -4·8 [IQR -11·1 to -2·5], p=0·031) and Nmen-dOMV (median titre change -12·6 [-33·1 to -8·5], p=0·031) for the six infants not colonised with Nlac (Figure 5A). Conversely, in the only infant persistently colonised with non-Y92-1009 Nlac, anti-Nlac-dOMV IgG titre increased (14·4 to 18·0; Figure 5A, pink datapoints), concomitant with an almost four-times increase in anti-Nmen-dOMV IgG titre (11·0 to 42·1; Figure 5B, pink datapoints).

Maternal and venous sampling at Visit 6 (15±2 weeks) was incorporated by study amendment (Appendix 5, p6). Both umbilical cord (Visit 3) and maternal venous (Visit 6) blood samples were available for 9/17 (53%) inoculated mothers completing all six study visits, of whom eight became colonised with Nlac Y92-1009 following inoculation. Anti-Nlac-dOMV IgG titre increased numerically in 7/8 (88%) of these women (median change 3·2 [IQR 0·9 to 10·2], p=0·055; Figure 5C), whereas there were trends towards decreased anti-Nmen-dOMV IgG titre in inoculated women (-2·6 [-16·0 to 10·7], p=0·55; Figure 5D), and decreased IgG titres against Nlac-dOMV (-3·4 [-8·2 to -2·0], p=0·063; Figure 5E) and Nmen-dOMV (-4·9 [-19·8 to 3·2], p=0·44; Figure 5F) in uninoculated women, although these changes were not significant.

Discussion

This study demonstrates that nasal Nlac Y92-1009 inoculation at 36-38 weeks' gestation induces sustained maternal but not subsequent infant or sibling colonisation or serological response by 15±2 weeks postpartum, despite evidence of mother-to-infant Mcat transmission, and of naturally-acquired Nlac transmission from siblings to their mothers. The inoculation strain was detected in only one infant and only at birth, with no associated serological response. Rather than true colonisation, this likely represents transient contribution to the infant's pioneer microbiome, the rapid influx of maternal and environmental microbes at birth, much of which is lost during niche-differentiation over the first days of life.[24]

These findings challenge conventional perceptions of infants as passive recipients of maternal microbes, and suggest that mother-to-infant URT microbial transmission is selective, and species-specific or even strain-specific. This has previously been suggested by observational microbiome research, with 95% of infant oral strains accounting for only 7% of maternal oral bacterial abundance at 3 days postpartum,[24] and just 13 maternal strains making up over 85% of infant oral abundance by 12 months postpartum.[25] However, this has hitherto not been demonstrated in an interventional respiratory study. It is unclear why a small subset of maternal URT strains appears disproportionately important in seeding the infant URT microbiome, while other microbes such as Nlac do not appear to transmit efficiently. There is evidence that immunisation during pregnancy against pathobionts like *N. meningitidis*, *S. pneumoniae* or *H. influenzae* can lead to increased infant antibody concentrations, although the impact of transplacental antibodies on commensal or pathobiont infant carriage remains unknown.[26] Alternatively, transmission success may be related to infant microbiome niche differentiation, including microbe-microbe and microbe-host interactions. Future investigations will include microbiome analyses in mother-infant pairs.

By including women and siblings naturally-colonised with Nlac, this study meaningfully builds on the literature to date. Baseline Nlac carriage in women with 1-5-year-old children was 41% (7/17), higher than previously reported (10% in women over 20 years old) and comparable with colonisation rates in young children,[7] suggesting child-to-mother transmission. Non-Y92-1009 Nlac colonisation and serological response was seen in one infant, associated with a four-times increase in cross-reactive anti-Nmen-dOMV IgG. However, it is not possible to determine if Nlac

was acquired from this infant's mother or sibling, or the timing of Nlac acquisition in any naturally-colonised uninoculated mothers or siblings.

Although sustained mother-to-infant inoculation-induced Nlac transmission was not seen, this proof-of-concept study showed that CHIM in pregnancy can be feasible, and the inoculation strain proved safe, genetically stable, and efficient at colonising inoculated women. There were no AEs related to study participation, and only minor longitudinal microevolution was seen over 21 weeks following inoculation. Nlac was detected at least once in 75% of women sampled after inoculation, and in 100% of those who received no antibiotics, but Nlac colonisation was reduced following antibiotics. As women likely to require intrapartum antibiotics were excluded at screening, antibiotic use may have been even higher in an unscreened population, with worldwide antibiotic exposure in pregnancy ranging from 20% to 65%.[27] Further, the confounding effect of antibiotics makes it difficult to comment on the effect of pregnancy per se on Nlac CHIM, compared with past studies in non-pregnant adults. Of note, no antibiotics were received by five of the mother-infant pairs in whom Mcat, but not Nlac, transmission was observed. This suggests that antibiotic exposure does not explain these apparent differences in Nlac and Mcat strain-sharing.

Comparisons with past Nlac CHIM studies are further limited by the small number of paired serum samples available. Lack of a pre-inoculation maternal serum sample and use of umbilical cord blood as an imperfect proxy for maternal serological status likely means that baseline maternal antibody titres were overestimated (Appendix 5, p6). This may explain why the previously-described increase in anti-Nmen-dOMV IgG induced by Nlac inoculation was not seen in this study (Figure 5D).[15] All seven infants sampled at Visit 6 had received the first dose of 4CMenB meningococcal serogroup B vaccine (routinely given at 8 weeks postnatally), complicating interpretation of infant serological analyses at this timepoint (Appendix 5A and 5B, p6). Additionally, Nlac colonisation density was quantified using oral swab culture results in this study, as opposed to nasal wash in previous Nlac CHIM studies.

The pragmatic sample size was based on the primary endpoint of detecting mother-to-infant Nlac Y92-1009 transmission, but downstream serological and genomic analyses were likely under-powered. Sample acquisition and processing methods may have led to an underestimation in Nlac and Mcat carriage in certain contexts: siblings were sampled only once,

Mcat was opportunistically detected on Nlac-selective agar, and oropharyngeal sampling was not performed in infants or siblings. However, edentulous pre-weaned infants are known to have reassuringly similar oropharyngeal and saliva microbiome profiles, and niche differentiation between adjacent anatomical sites remains incomplete during infancy.[28,29] Furthermore, Nlac was detected in saliva from 5/12 (41.7%) sampled siblings, which is comparable with oropharyngeal carriage rates previously reported in this age-group.[6–8]

Despite stated limitations, this proof-of-concept trial will be a pathfinder for future interventional studies investigating infant respiratory commensalisation. Although Nlac inoculation in pregnancy does not appear to result in infant colonisation, future trials may involve maternal inoculation with other health-associated commensals that appear to transmit naturally from mother-to-infant (such as *Dolosigranulum* and *Corynebacterium* species), or even inoculation of co-habiting siblings or infants themselves.

Figure legends

Figure 1. Study overview.

A) Study timeline and participant procedures. w: weeks (gestational age for Visits 1 and 2; postpartum age for Visits 3 to 6); d: days; h: hours; S: saliva; N: nasopharyngeal; O: oropharyngeal; UC: umbilical cord; IV: infant venous; MV: maternal venous; (+): optional (refusal or inability to provide a sample did not impact study participation).

B) Participant flow diagram. GBS: group B streptococcus.

C) Visit timing relative to birth and inoculation. Red lines indicate median and interquartile range; V: Visit.

Figure 2. Neisseria lactamica (Nlac) colonisation kinetics in inoculated and naturally-colonised participants and their children.

CFU: colony-forming units; h: hours; d: days; w: weeks. Error bars (B and D) indicate median and interquartile range. Visits 3, 4, 5 and 6: 0-24h, 7±3d, 28±3d and 15±2w postpartum, respectively.

A) Nlac colonisation detected by selective culture of oropharyngeal (maternal) and saliva (infant and sibling) samples obtained at study visits.

B) Nlac colonisation density in inoculated participants was lower in those who received antibiotics (red) compared with those who received none (green). Significance assessed using Mann-Whitney test.

C) Significant inverse correlation seen between Nlac colonisation density and elapsed time following inoculation for all inoculated volunteers completing the study (Spearman's rho [r_s]-0·25, p=0·037; black), and for those who received no antibiotics (r_s-0·55, p=0·0005; green), but not those who receive antibiotics (r_s-0·11, p>0·05; red).

D) Trend towards lower median Nlac colonisation density in naturally-colonised compared with inoculated participants at each postpartum study visit (p>0·05 for all). Significance assessed using Mann-Whitney test.

Figure 3. Whole genome sequencing of Neisseria lactamica (Nlac) isolates derived from inoculated and naturally-colonised women and their children.

Sequencing (NovaSeq 6000, Illumina) performed on up to three Nlac isolates per sample for the first and final two samples that were culture-positive for each participant. Additional long-read sequencing (GridION, Oxford Nanopore Technologies) and hybrid assembly performed on one isolate from each naturally-colonised woman (7 isolates). Multiple sequence alignment and variant calling performed using Snippy, with reference genome Nlac Y92-1009 (BioProject accession PRJNA331097), and correction for putative recombination using Gubbins (Appendix 3, p4). Total variants: insertions, deletions and substitutions; SNP: single nucleotide polymorphism; RC: recombination-corrected. M: mother; C: under five-year-old child (sibling); I: infant; inoc: Nlac Y92-1009 inoculum. Sequence names comprising unique sequence identifier, family number, participant type, and visit number (e.g. 239590_24M4: sequence 239590 derived from a maternal sample from family 24 at Visit 4).

A) Summary of Nlac isolates frozen and sequenced for each participant group.

B) Nlac Y92-1009 (inoculation strain) genomes were highly similar across participants and timepoints. Error bars indicate median and interquartile range.

C) Significant correlation between variant count and elapsed time following inoculation, for total variants (Spearman's rho [r_s] 0·72, p<0·0001; green), total SNPs (r_s 0·50, p<0·0001, pink), and recombination-corrected SNPs (r_s 0.32, p=0·0010; blue). Line of best fit based on simple linear regression model.

D) Heatmap showing recombination-corrected SNP distance between pairs of Nlac genomes, with each small square representing a different genome. Colours used to distinguish between inoculated (blue) and uninoculated (red, orange, green, pink, purple, turquoise) families.

E) Nlac phylogenetic tree derived from multiple sequence alignment. Constructed using RAxML-NG and visualised with Interactive Tree of Life. Branch length is proportional to phylogenetic distance, indicated by scale numbers (variants per site). Colours used to distinguish between inoculated (blue) and uninoculated (red, orange, green, turquoise, purple, pink) families.

Figure 4. Whole genome sequencing of Moraxella catarrhalis (Mcat) isolates derived from inoculated and naturally-colonised women and their children.

Sequencing (NovaSeq 6000, Illumina) performed on up to three Mcat isolates per individual for any families where at least two individuals were colonised with Mcat. Multiple sequence alignment and variant calling performed using Snippy, with reference genome Mcat CCRI-195ME (BioProject accession PRJNA350869), and correction for putative recombination using Gubbins (Appendix 3, p4). SNP: single nucleotide polymorphism; M: mother; C: under five-year-old child (sibling); I: infant; O: oropharynx; S: saliva; N: nasopharynx; B: breastmilk. Sequence identifiers comprise family number, sample type, and visit number (e.g. 18IN6: sequence derived from an infant nasopharyngeal sample from family 18 at Visit 6).

A) Summary of Mcat isolates frozen and sequenced for each participant group.

B) Heatmap showing recombination-corrected SNP distance between pairs of Mcat genomes, with each small square representing a different genome. Evidence of probable (0-10 SNPs) and possible (10-100 SNPs) strain-sharing in mother-infant pairs from the same family (green and blue, respectively) and from different families (red and orange, respectively; dashed red line used for non-adjacent sequences); and between related sibling-infant pairs (pink, 0-10 SNPs).

Figure 5. Changes in serum immunoglobulin G (IgG) titres against *Neisseria lactamica* (Nlac) and *N. meningitidis* (Nmen).

IgG against deoxycholate-extracted outer-membrane vesicles derived from Nlac Y92-1009 (Nlac-dOMV; A, C, E) and Nmen H44/76 (Nmen-dOMV; B, D, F) in serum from umbilical cord, infant venous (A and B) and maternal venous (C, D, E, F) blood samples. Serum titres were measured by enzyme-linked immunosorbent assay (ELISA), through interpolation compared to a reference serum from a single human donor. Datapoint colours indicate participant colonisation status (black: uncolonised; blue: Nlac Y92-1009-colonised; pink: Nlac non-Y92-1009-colonised). Error bars indicate median titre and interquartile range, with median values compared across timepoints using Wilcoxon matched-pair signed rank test. *Results from the only infant persistently colonised with Nlac non-Y92-1009 are indicated in pink, but are not included in statistical analysis of titre change in uncolonised infants. Paired sera (Visit 3 and Visit 5) were not available from the only infant in whom Nlac Y92-1009 was identified at birth.

Contributors

AAT, CEJ, RCR, JRL, DWC, APD and DB conceived of and designed the trial, with AAT as Principal Investigator and CEJ and RCR as joint Chief Investigators. AAT and CEJ wrote the study protocol, with input from RCR, JRL, DWC, APD and DB. AAT, JRL and JMG produced the batch of Nlac inoculum. AAT carried out the clinical study visits, with support from clinical fellows and trial assistants. AAT and LR performed microbiological analyses to identify Nlac and Mcat, and prepared isolates for whole genome sequencing, with support from laboratory technicians. DFG optimised the anti-Nlac- and anti-Nmen-dOMV serum ELISA; JMG and APD performed the ELISAS; AAT and APD analysed and verified the ELISA data. AAT collated and analysed all clinical, microbiological and sequencing data. AAT and CEJ verified the data and wrote the manuscript first draft, and all authors contributed to critically appraising and revising the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of Interests

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Data Sharing

The full study protocol can be accessed online (https://eprints.soton.ac.uk/492361/). All whole genome sequences derived from Nlac isolates have been uploaded to PubMLST.org, a collection of curated open-access databases for molecular typing and microbial genome diversity. Requests for additional deidentified participant data will be reviewed by the authors, and data sharing may be permitted to achieve the aims in the approved proposal, following appropriate institutional and ethical review. Data requests should be directed to the

corresponding author, and requestors will be required to sign a data access agreement. Data will be available beginning one month and ending 24 months after publication of this Article.

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Appendices

Table 1. Participant characteristics and clinical data

Screening (Visit 1; n=31 women)		
Median age (range)		33·5 (23·1-39·9)
Ethnicity (%)	White, British	26 (83·9)
	White, any other White background	3 (9·7)
	Asian, Pakistani	1 (3·2)
	Other, any other ethnic group	1 (3·2)
Household contact with children 1-5 years old (%)		17 (54·8)
Household contact with animals (%)		16 (51·6)
Birth (Visit 3; n=28 mother-infant pairs)		
Median gestational age in weeks at delivery (range)		40·0 (38·0-42·0)
Mode of delivery (%)	Vaginal	25 (89·3)
	Caesarean	3 (10·7)
	Forceps or ventouse (at any time during labour)	6 (21·4)
	Birthing pool (at any time during labour)	5 (17·9)
Peripartum (labour to 24 hours postpartum) systemic (oral/parenteral) antibiotics (%)	Maternal before cord clamping only	3 (10·7)
	Maternal after cord clamping only	7 (25)
	Maternal before and after cord clamping	1 (3·6)
	Infant	2 (7·1)
Completed all follow-up (Visit 6; n=24 mother-infant pairs)		
Any household contact with smoker (%)		4 (16·7)
Any systemic (oral/parenteral) antibiotics during study (%)	Maternal	13 (54·2)
	Before birth	6 (25.0)
	After birth	9 (37.5)
	Infant	1 (4·2)
Mode of feeding (%)	Any breastmilk	21 (87·5)
	Exclusively breastmilk	13 (54·2)
	Any formula	11 (45·8)
	Exclusively formula	3 (12·5)

Figure 1A

	Screening	Inoculation	Birth	Post-partum follow-up visits		
	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
	34-37w	36-38w	0-24h	7±3d	28±3d	15±2w
Maternal swabs (S, N, O)	+	+	+	+	+	+
Infant swabs (S, N)			+	+	+	+
Sibling swabs (S)						(+)
Breastmilk			(+)	(+)	(+)	(+)
Blood			(UC)		(IV)	(IV, MV)
Questionnaire	(+)				(+)	

Figure 1B

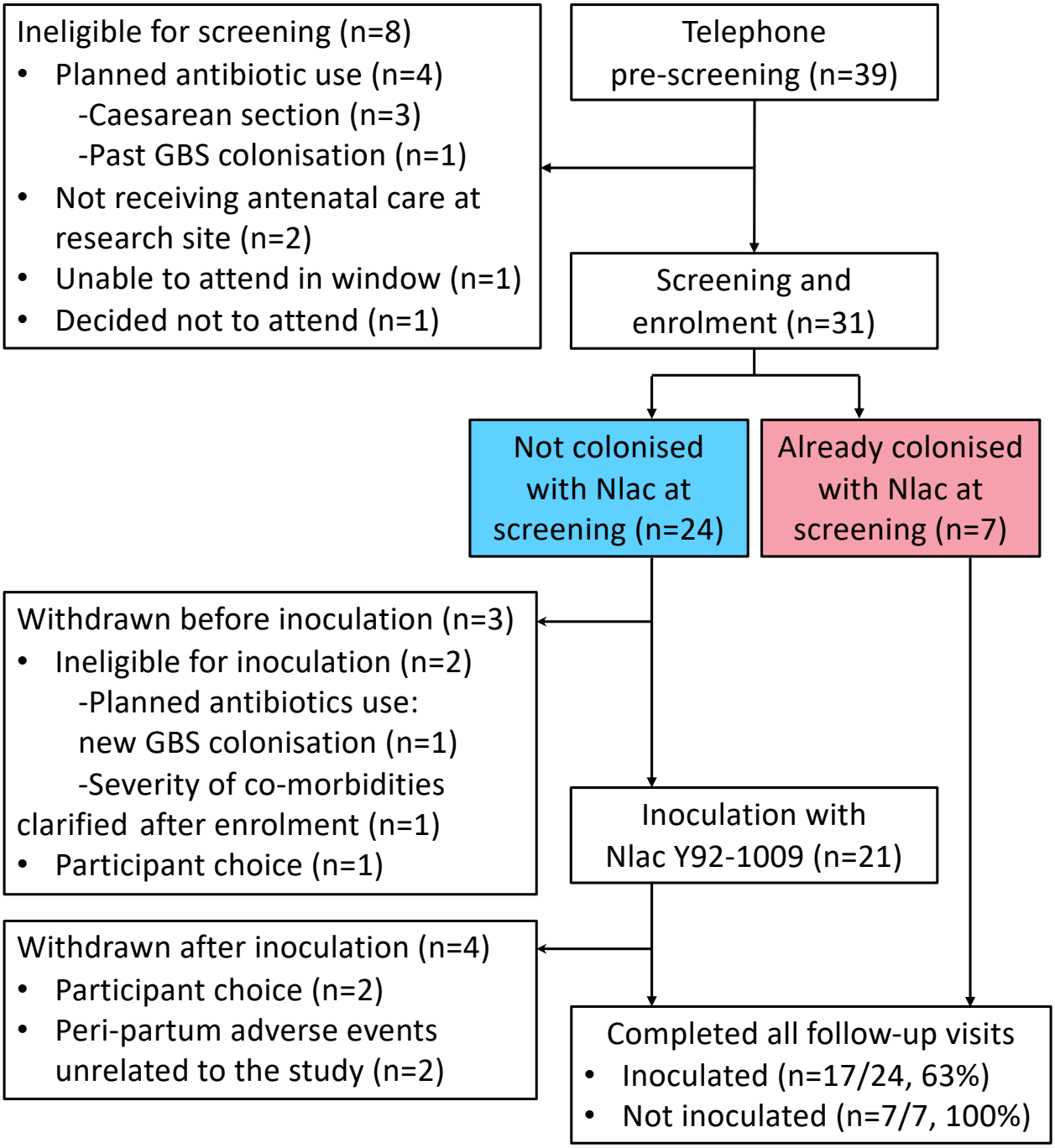

Ineligible for screening (n=8)
• Planned antibiotic use (n=4)
-Caesarean section (n=3)
-Past GBS colonisation (n=1)
• Not receiving antenatal care at research site (n=2)
• Unable to attend in window (n=1)
• Decided not to attend (n=1)
Telephone pre-screening (n=39)
Screening and enrolment (n=31)
Not colonised with Nlac at screening (n=24)
Already colonised with Nlac at screening (n=7)
Withdrawn before inoculation (n=3)
• Ineligible for inoculation (n=2)
-Planned antibiotics use: new GBS colonisation (n=1)
-Severity of co-morbidities clarified after enrolment (n=1)
• Participant choice (n=1)
Inoculation with Nlac Y92-1009 (n=21)
Withdrawn after inoculation (n=4)
• Participant choice (n=2)
• Peri-partum adverse events unrelated to the study (n=2)
Completed all follow-up visits
• Inoculated (n=17/24, 63%)
• Not inoculated (n=7/7, 100%)

Figure 1C

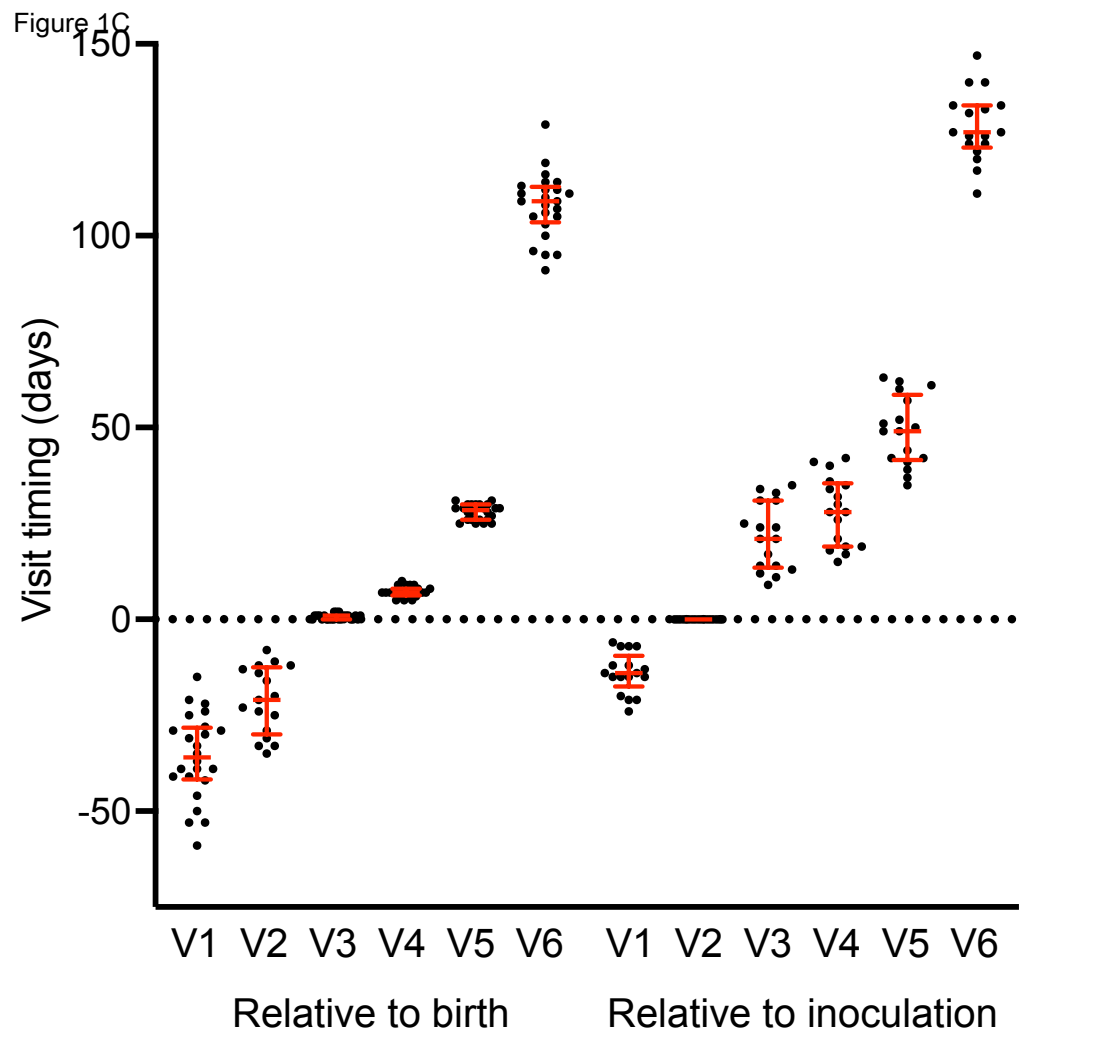

150
100
50
0
-50
Visit timing (days)
V1
V2
V3
V4
V5
V6
V1
V2
V3
V4
V5
V6
Relative to birth
Relative to inoculation

Figure 2A

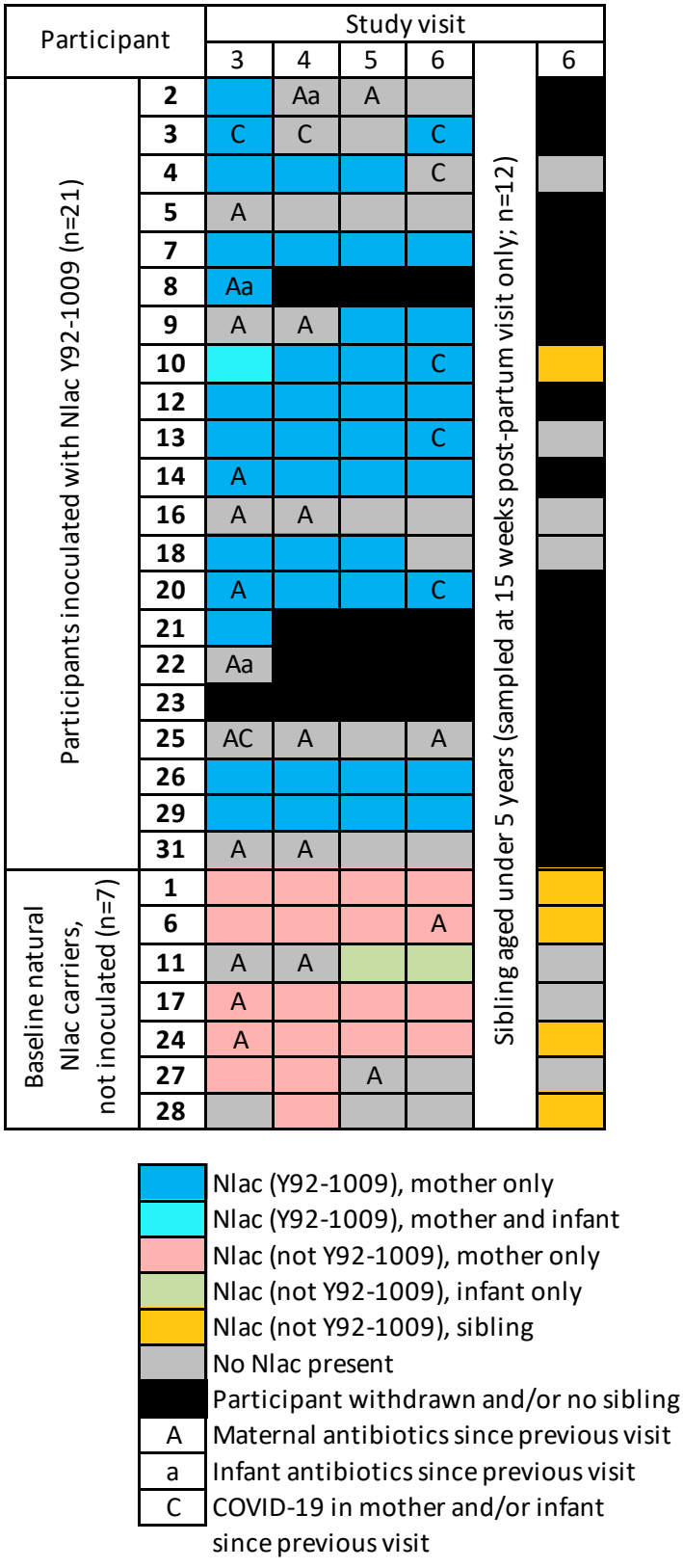

Participant
Study visit
3
4
5
6
6
Participants inoculated with Nlac Y92-1009 (n=21)
2 Aa A
3 C C C
4 C
5 A
7
8 Aa
9 A A
10 C
12
13 C
14 A
16 A A
18
20 A C
21
22 Aa
23
25 AC A A
26
29
31 A A
Baseline natural Nlac carriers, not inoculated (n=7)
1
6 A
11 A A
17 A
24 A
27 A
28
Sibling aged under 5 years (sampled at 15 weeks post-partum visit only; n=12)
Nlac (Y92-1009), mother only
Nlac (Y92-1009), mother and infant
Nlac (not Y92-1009), mother only
Nlac (not Y92-1009), infant only
Nlac (not Y92-1009), sibling
No Nlac present
Participant withdrawn and/or no sibling
A Maternal antibiotics since previous visit
a Infant antibiotics since previous visit
C COVID-19 in mother and/or infant since previous visit

Figure 2B

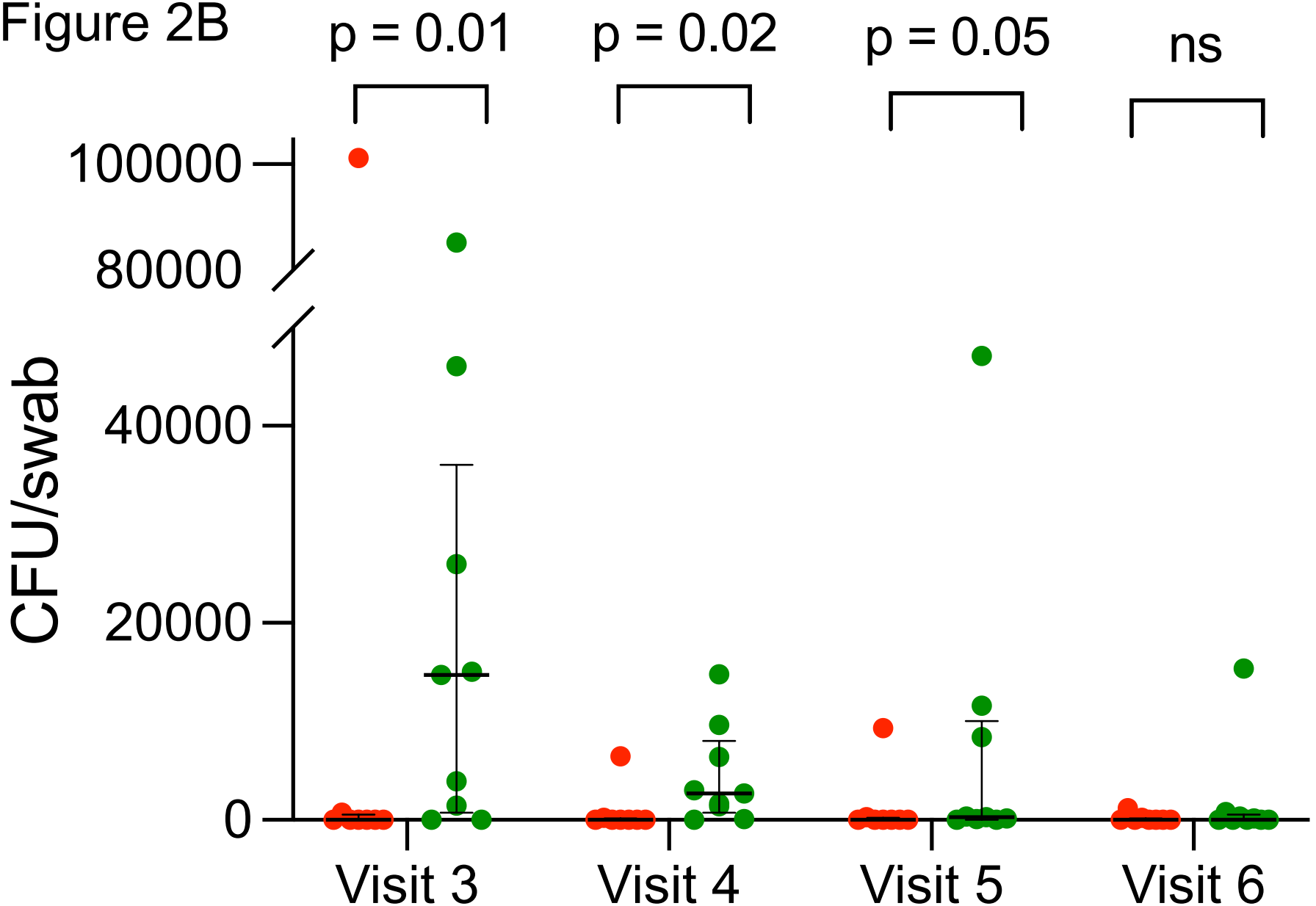

p = 0.01
p = 0.02
p = 0.05
ns
100000
80000
40000
20000
0
CFU/swab
Visit 3
Visit 4
Visit 5
Visit 6

Figure 2C

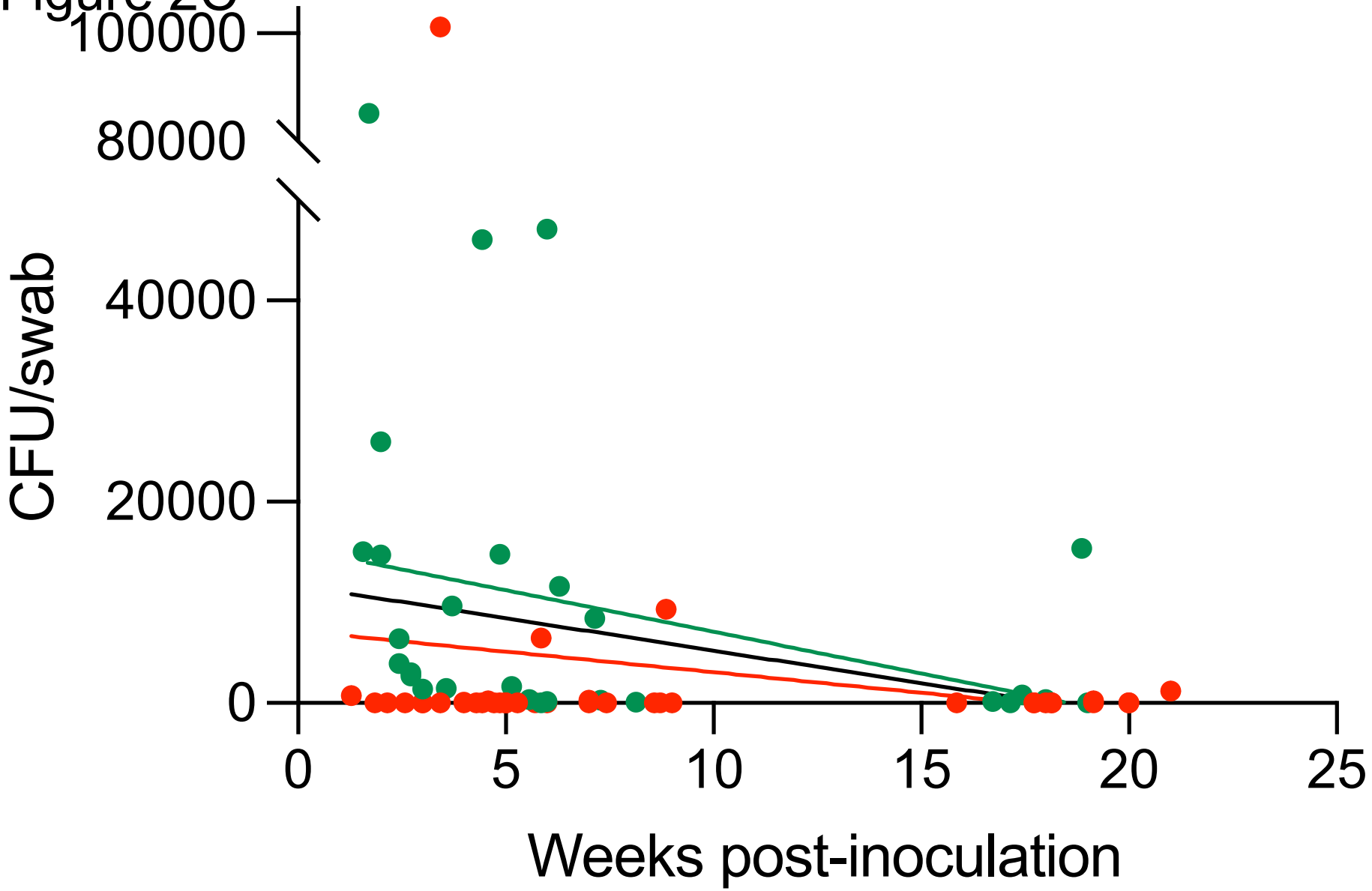

100000
80000
40000
20000
0
CFU/swab
0
5
10
15
20
25
Weeks post-inoculation

Figure 2D

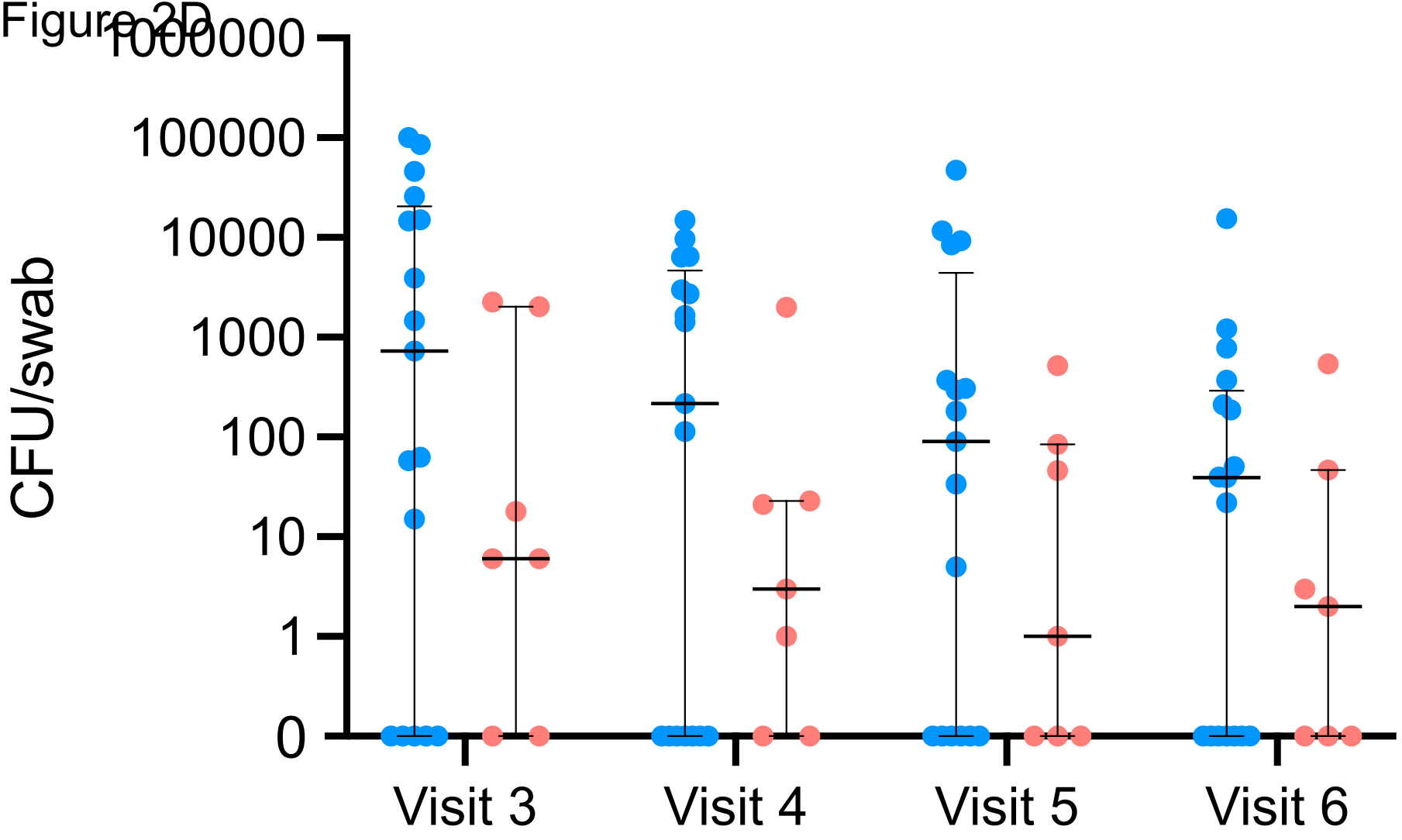

1000000
100000
10000
1000
100
10
1
0
CFU/swab
Visit 3
Visit 4
Visit 5
Visit 6

Figure 3A

Participant group (n)	Number of isolates	
Inoculated	**Frozen**	**Sequenced (% of frozen)**
Mother (15)	223	105 (47)
Infant (1)	5	3 (60)
Sibling (1)	5	3 (60)
Uninoculated		
Mother (7)	115	54 (47)
Infant (1)	10	6 (60)
Sibling (4)	20	12 (60)

Figure 3B

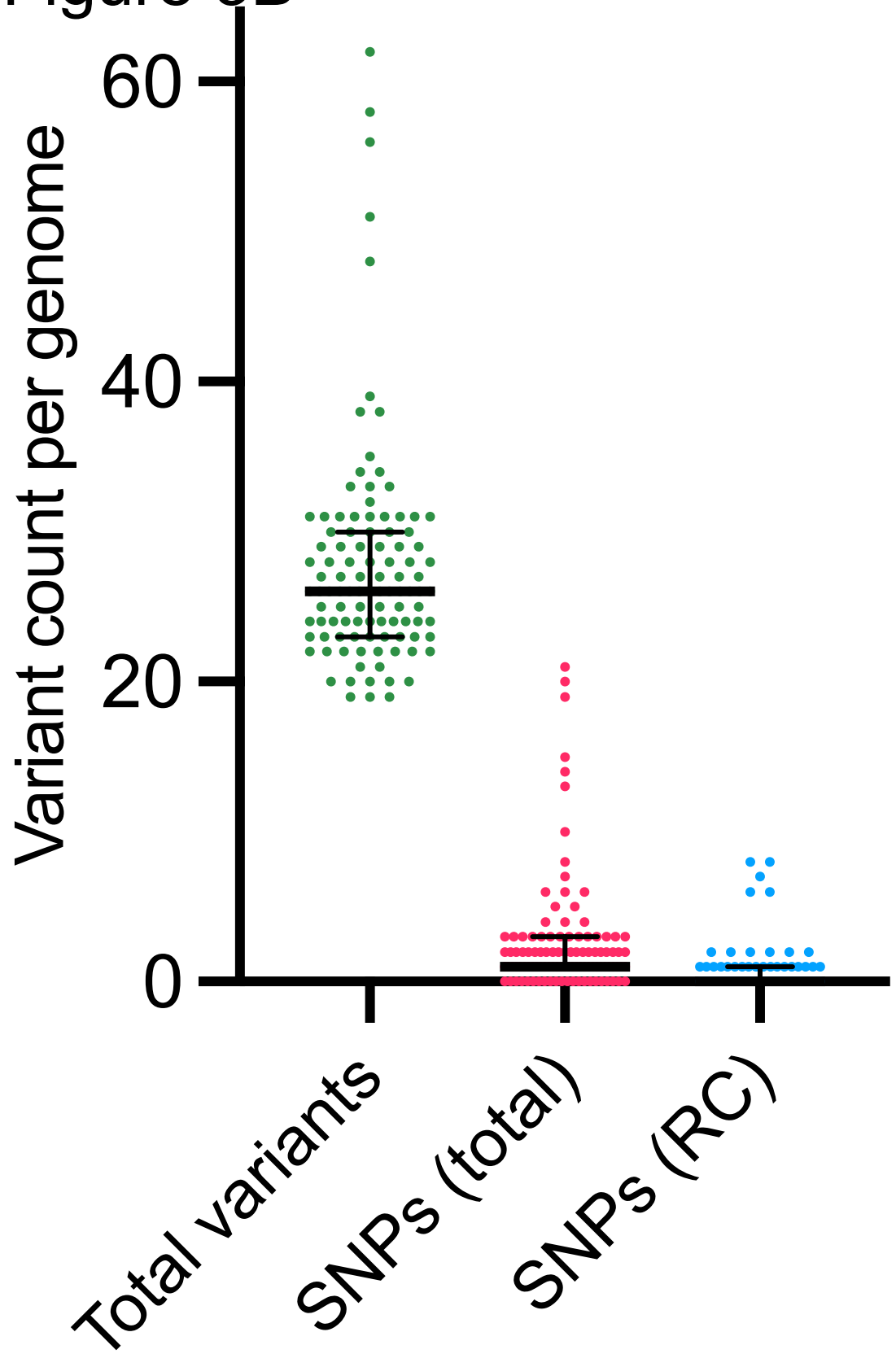

Variant count per genome
60
40
20
0
Total variants
SNPs (total)
SNPs (RC)

Figure 3C

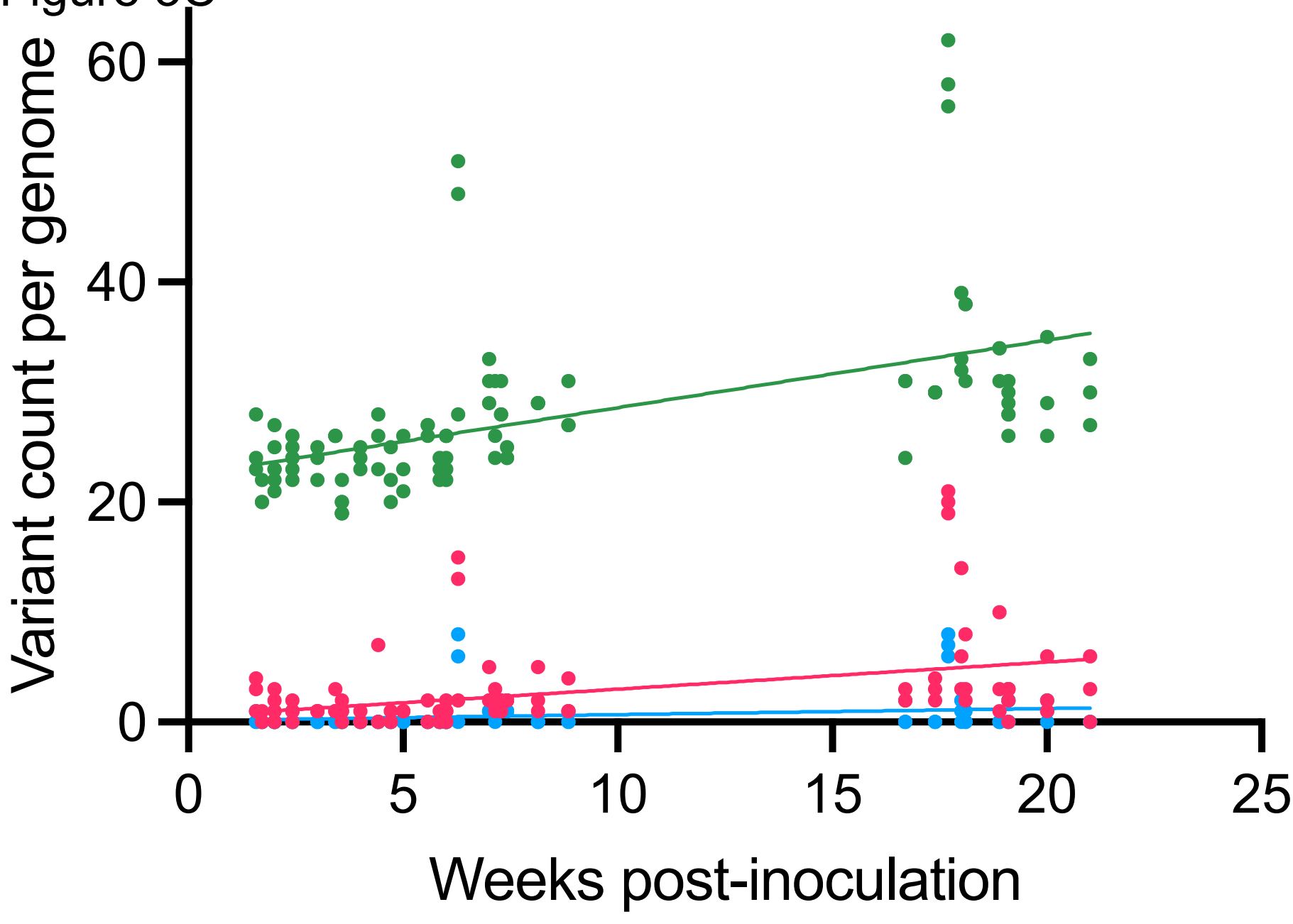

Variant count per genome
60
40
20
0
0
5
10
15
20
25
Weeks post-inoculation

Figure 3D

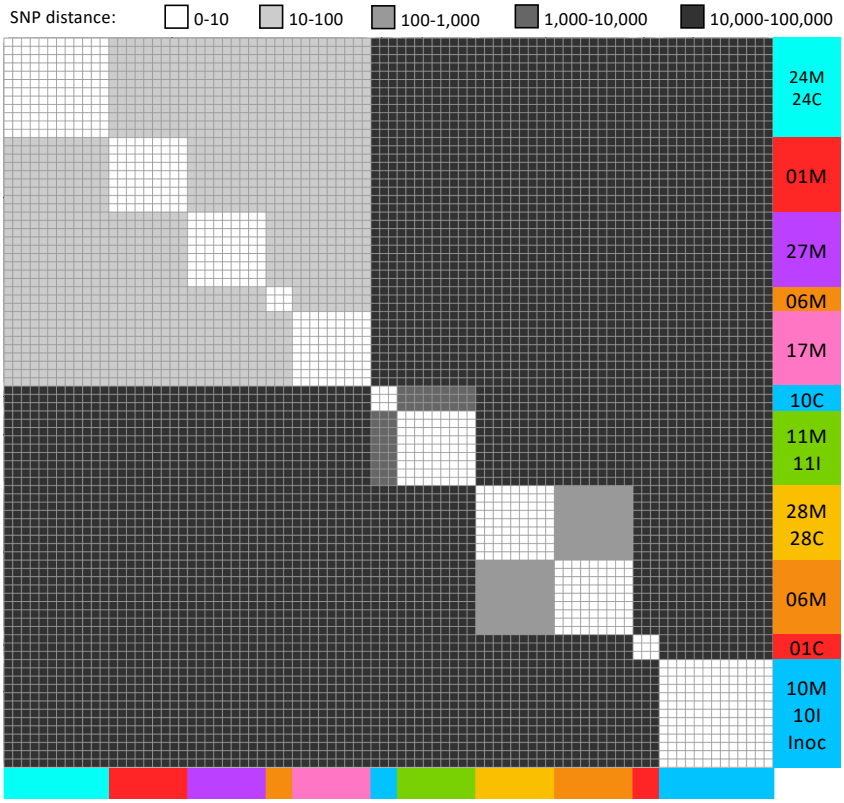
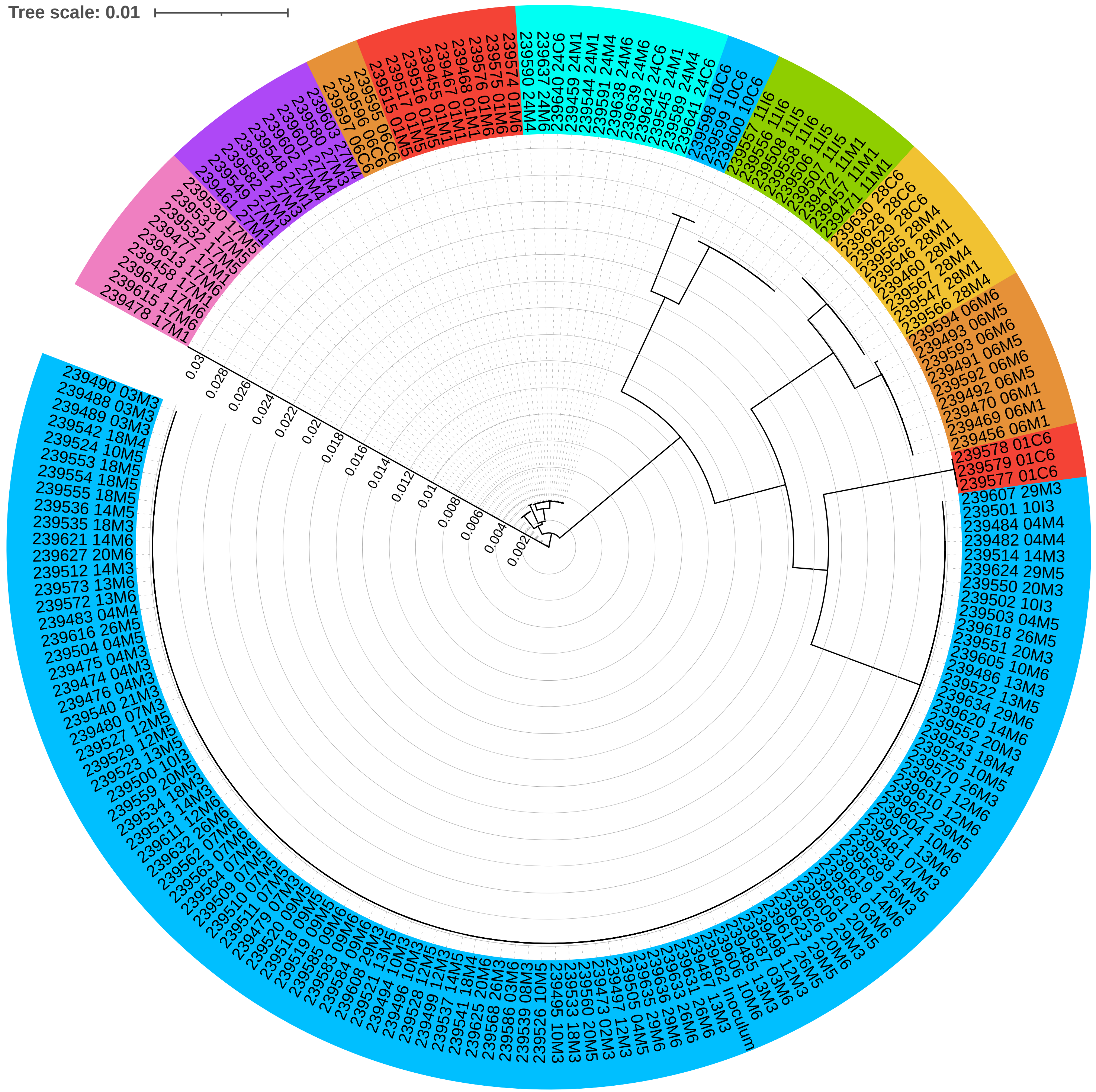
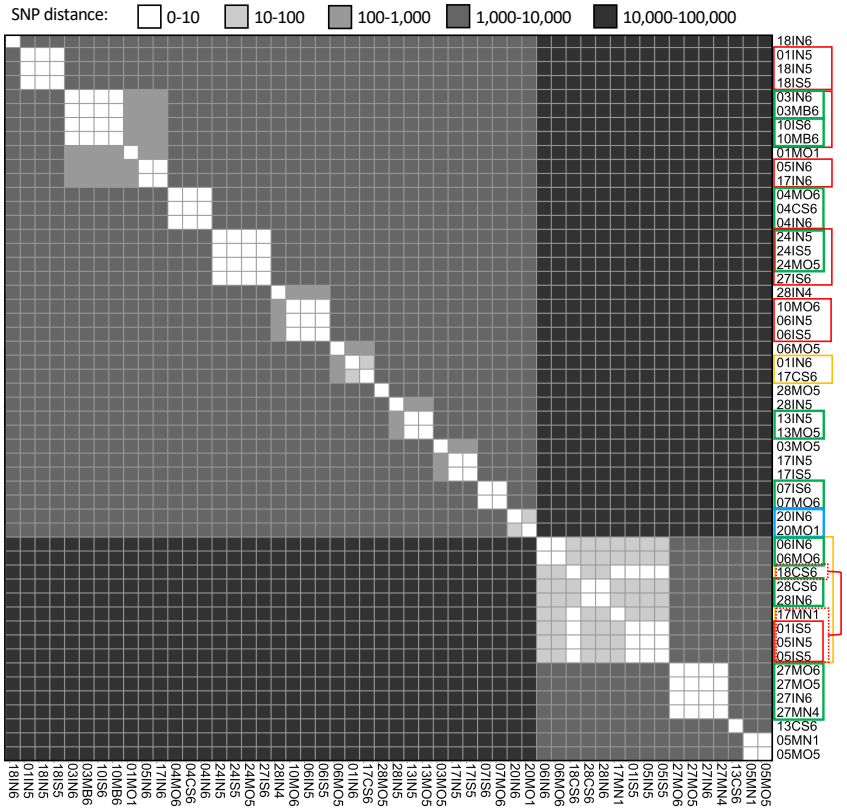

SNP distance:
0-10
10-100
100-1,000
1,000-10,000
10,000-100,000
24M
24C
01M
27M
06M
17M
10C
11M
11I
28M
28C
06M
01C
10M
10I
Inoc

Figure 3E


Tree scale: 0.01
0.002
0.004
0.006
0.008
0.01
0.012
0.014
0.016
0.018
0.02
0.022
0.024
0.026
0.028
0.03

Participant group (n)	Number of isolates	
Inoculated	**Frozen**	**Sequenced (% of frozen)**
Mother (7)	15	10 (67)
Infant (8)	28	13 (46)
Sibling (3)	3	3 (100)
Uninoculated		
Mother (6)	11	9 (82)
Infant (6)	16	15 (94)
Sibling (2)	2	2 (100)

Figure 4B


SNP distance:
0-10
10-100
100-1,000
1,000-10,000
10,000-100,000
18IN6
01IN5
18IN5
18IS5
03IN6
03MB6
10IS6
10MB6
01MO1
05IN6
17IN6
04MO6
04CS6
04IN6
24IN5
24IS5
24MO5
27IS6
28IN4
10MO6
06IN5
06IS5
06MO5
01IN6
17CS6
28MO5
28IN5
13IN5
13MO5
03MO5
17IN5
17IS5
07IS6
07MO6
20IN6
20MO1
06IN6
06MO6
18CS6
28CS6
28IN6
17MN1
01IS5
05IN5
05IS5
27MO6
27MO5
27IN6
27MN4
13CS6
05MN1
05MO5