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Real-world solutions for real-world settings: Effects of storage conditions on Streptococcus pneumoniae detection

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Abstract

Background

Appropriate storage and transport conditions are crucial for ensuring bacterial viability within biological specimens. Nasopharyngeal carriage studies are common for *Streptococcus pneumoniae*. However, storing or transporting specimens at ultra-low temperatures as recommended by the World Health Organization is not possible in all settings. Here, we assess the impact of alternative storage conditions on pneumococcal viability and density using laboratory-prepared samples.

Methods

Flocked swabs were inoculated with low (n = 395) or medium (n = 395) loads of single pneumococcal isolates. Twenty different serotypes were tested. Swabs were stored in skim milk, tryptone, glucose and glycerol (STGG) medium at -80°C (gold standard), -20°C and 4°C, and in silica desiccant packets (SDPs) at 4°C and 30°C. Viable counts were performed at inoculation (baseline), and 2, 7, 14, 21 and 28 days post storage. A subset of samples from baseline and 28 days post storage were assessed for pneumococcal detection and density using culture-independent, molecular methods.

Results

Using culture-based methods, pneumococcal recovery was high (≥ 80%) up to 28 days when samples were stored at -20°C and − 80°C in STGG. Samples stored at 4°C in both STGG and SDPs had inconsistent recovery (< 80%) by 28 days, and samples stored at 30°C in SDPs had very low recovery (< 15%) at all timepoints. Overall, when compared by culture to the inoculum load, density was substantially reduced when alternative storage conditions were used. When density was assessed by *lytA* qPCR, pneumococci could be detected for most (99%) samples in select storage conditions.

Conclusions

Improper storage of specimens may lead to underestimations of pneumococcal prevalence and density, especially when culture-based methods are used. Given this, study outcomes and methods of testing should be considered when determining feasible transport and storage conditions. We identified alternative storage conditions to the gold standard that could enable studies in remote or complex settings.

Introduction

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium with > 100 pneumococcal capsular serotypes[3,4]. It commonly colonises the nasopharynx (termed ‘carriage’), particularly in young

children[1,2]. Pneumococcal carriage is considered a prerequisite to disease[2] and increased pneumococcal density is associated with severe pneumonia[5,6]. Pneumonia and invasive pneumococcal disease (IPD) such as meningitis and sepsis are a leading cause of childhood mortality, particularly in low- and middle-income countries (LMICs)[7]. Carriage studies are important to monitor the pneumococci circulating in a population, and assess herd protection as a proxy for disease, where IPD surveillance is challenging[2]. Studies investigating pneumococcal prevalence rely on appropriate methods to collect, store and transport specimens to monitor circulating serotypes.

The World Health Organization (WHO) guidelines for pneumococcal carriage studies recommend placing nasopharyngeal swabs in skim milk, tryptone, glucose and glycerol (STGG) medium and storing at ultra-low temperatures (ULT) (typically between − 70°C and − 80°C for biological specimens) as soon as possible after collection[8]. However, in some LMICs, remote settings, humanitarian crises[9,10] or during other large-scale disruptions it can be difficult to apply these standards. Challenges can include a lack of access to dry ice or ULT freezers, or difficulties with equipment maintenance and irregular power supplies[11]. Accurate and practical approaches for sample collection and pneumococcal detection are essential for generating reliable data to inform vaccine-decision making especially in high disease burden or challenging settings. Silica desiccant packets (SDPs) have been found to be an effective alternative to STGG for transporting specimens and isolates[12] for delayed culture of several bacterial species including the pneumococcus[13]. Here, we examined the impact of alternative storage conditions on the detection of pneumococcal prevalence and density in laboratory-prepared samples, to identify viable alternative storage conditions for pneumococcal carriage studies specimen storage.

Methods

Preparation of laboratory-prepared nasopharyngeal samples

Twenty different *S. pneumoniae* isolates, each representing a different serotype (Supplementary Table 1), were used to create laboratory prepared medium (10^4 – 10^5 colony forming units (CFU)/ml) and low (10^2 – 10^3 CFU) bacterial load samples for each serotype. Nylon FLOQSwabs (Copan Diagnostics, USA) were each inoculated with 10 µl of one pneumococcal stock and stored directly in 1 ml of STGG at -80°C (gold standard; n = 30), -20°C (domestic freezer, Samsung; n = 190) and 4°C (domestic refrigerator, Samsung; n = 190), or into SDPs at 4°C (domestic refrigerator, Samsung; n = 190), and at 30°C (dry incubator, Ratek; n = 190) to mimic potential field site conditions. All storage conditions were tested concurrently, with samples recovered at 2, 7, 14 and 21 days for all serotypes (n = 20). Most samples (n = 15 serotypes) were tested to an extended timepoint of 28 days to reflect extended delays in sample processing (Supplementary Fig. 1).

Recovery and enumeration of pneumococci

Viable counts were performed at baseline (inoculation, day 0) and each timepoint (2, 7, 14, 21 and 28 days post-storage) following swab recovery for all conditions. Frozen STGG samples were placed on wet ice until thawed. Swabs stored in SDPs were first removed carefully from the SDPs, then placed into 1 ml STGG on wet ice prior to culture. To perform viable counts, all samples were vortexed thoroughly before 50 µl was plated on horse blood agar (HBA) and incubated overnight at 37°C, 5% CO_2. The next day pneumococci were enumerated. Growth of at least one colony on recovery was reported as viable, corresponding to a limit of detection of 20 CFU/ml. Culture-based density was reported as $\log_{10}$ CFU/ml.

Molecular detection of pneumococci

To determine whether molecular methods could reliably detect pneumococci from these alternative storage conditions DNA was extracted from 100 µl STGG from selected samples. Isolates at inoculum and at the 28 days post storage timepoint for the gold standard and best and poorest performing methods by culture (n = 120 samples total) were selected for molecular analysis. DNA was extracted using the QIAcube HT instrument as previously described[12]. Similar to previous studies[14,15], pneumococci were detected and quantified by quantitative real-time PCR (qPCR) targeting the *lytA* gene[16] using 5 µl template DNA and the AriaMX PCR instrument (Agilent). Molecular-based density was reported as $\log_{10}$ genome equivalents (GE)/ml, with a Cq < 35 considered positive.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8.1.1). The pneumococcal detection rate was reported as the percentage of samples with pneumococci detected at each timepoint. Density data were reported as mean $\log_{10}$ CFU/ml ± standard deviation (culture) or $\log_{10}$ GE/ml ± the standard deviation (*lytA* qPCR). The difference in mean and 95% CI was reported for lytA qPCR results. The overall pneumococcal detection rate at each timepoint post storage was compared to baseline (100% detection) using a Fisher's exact test. Density data were reported as mean $\log_{10}$ CFU/ml ± standard deviation (culture) or $\log_{10}$ GE/ml ± the standard deviation (*lytA* qPCR). For density analyses, each serotype was compared with the baseline inoculum using an ANOVA (only in pneumococcal positive samples) at each timepoint and compared using individual t-tests conducted. We determined *a priori* that a suitable method would result in a ≥ 80% detection rate across all timepoints, isolates and bacterial loads tested and ≤ 0.5 $\log_{10}$ loss in density compared to the baseline inoculum for both culture and molecular analyses.

Results

Pneumococcal detection and density by culture

Firstly, the effect of each storage condition on recovery of viable pneumococci was examined in samples which were stored to the 28 day post storage timepoint. The gold standard storage condition (-80°C in

STGG) allowed for recovery of pneumococcus from all samples at 28 days storage (30/30 (100%)). When samples were stored in STGG at -20°C, regardless of the initial bacterial load, the detection rate of recoverable pneumococci was ≥ 80% for up to 28 days post storage when compared to the baseline ($p = 0.07$ for low bacterial load, $p = 0.43$ for medium bacterial load) (Fig. 1A and B). At 4°C in STGG, 47% (7/15, $p = 0.0003$) and 74% (11/15, $p = 0.03$) of low and medium bacterial load samples were recovered respectively at 28 days post storage. Low load samples remained above the threshold up to 2 days and for medium load samples 7 days. For samples stored at 4°C in SDPs, 40% (6/15, $p < 0.0001$) of low bacterial load and 80% (12/15, $p = 0.07$) of medium bacterial load samples could be recovered after 28 days. Of these, recovery of low load samples remained above the threshold until 2 days, and medium density for 7 days. All samples stored at 30°C in SDPs were unable to be recovered after 28 days for both low and medium bacterial loads ($p < 0.0001$ for both loads); at no timepoint could samples stored under this condition be recovered at a rate above the 80% study threshold.

Next, we assessed the effect of storage conditions on pneumococcal density using culture-based methods. Density was not examined in culture negative samples. At baseline, low bacterial load samples had a mean pneumococcal density of 3.49 ± 0.36 $\log_{10}$ CFU/ml and medium bacterial load samples had a mean density of 5.04 ± 0.37 $\log_{10}$ CFU/ml for (Fig. 2A and 2B). All samples stored at 30°C in SDPs were non-viable at 28 days, and therefore density by culture could not be assessed for this condition. Regardless of initial bacterial load and storage condition, the mean density declined by > 0.5 $\log_{10}$ CFU/ml by 28 days for each of the assessable alternative storage conditions (Supplementary Table 3). When compared to the baseline inoculum the combined mean density of all samples with growth was substantially reduced for both low ($p = 0.0084$) and medium ($p = 0.0103$) bacterial load samples (Supplementary Table 2).

At 28 days, we also compared the pneumococcal density of samples stored in each of the alternative storage conditions to samples stored using the gold standard method (-80°C in STGG). The density of all medium bacterial load samples in all conditions declined substantially and by > 0.5 $\log_{10}$ CFU/ml over the time course ($p < 0.0001$ for each condition) and would be deemed unsuitable as per the study threshold. For low bacterial load samples, the density of samples stored at -20°C in STGG did not decrease significantly at 28 days ($p = 0.11$), however density decreased significantly for samples stored at 4°C in STGG, and 30°C and 4°C in SDPs ($p < 0.0001$ for all conditions).

Pneumococcal detection and density by molecular methods

For a subset of samples, following inoculation at baseline and recovery at 28 days (n = 15 samples per method, per bacterial load) we also assessed pneumococcal detection and density using *lytA* qPCR. We tested both low and medium bacterial load samples stored in 30°C SDPs (our poorest performing method by culture), -20°C STGG (our best performing method by culture) and the gold standard (-80°C STGG). Overall, for all samples tested, the pneumococcal detection rate was 99% (119/120). A low

bacterial load serotype 34 sample stored at 30°C in SDP for 28 days was the only sample found to be *lytA* qPCR negative.

Next, we assessed the effect of the selected storage conditions on pneumococcal density using *lytA* qPCR. To do this, we compared mean density data for each condition following 28 days storage to our baseline results. At baseline, low bacterial load samples had a mean pneumococcal density of 5.47 ± 0.35 $\log_{10}$ GE/ml and medium bacterial load samples had a mean density of 6.53 ± 0.40 $\log_{10}$ GE/ml ((Fig. 3A and 3B). The mean density of samples stored at -80°C and – 20°C in STGG at 28 days remained within the study threshold (< 0.5 $\log_{10}$ GE/ml loss in density) regardless of the initial bacterial load. The mean pneumococcal density of low bacterial load samples stored at -80°C in STGG, -20°C in STGG and 30°C in SDPs was similar to that of the baseline (p = 0.53, p = 0.06 and p = 0.22, respectively) and within the study threshold (Supplementary Table 4). For medium load samples, there were significant decreases in density of samples stored at 30°C in SDPs (p = 0.0006) which failed to meet the study threshold.

We also compared the density of samples stored in each alternative storage conditions to that of the gold standard (-80°C in STGG) at 28 days. Storage at -20°C in STGG, regardless of initial bacterial load, was within the study threshold (Supplementary Table 4). However, storage at 30°C in SDPs resulted in significant losses in density for medium bacterial load samples which failed to meet the study threshold.

Discussion

For pneumococcal carriage studies, the current WHO guidelines recommend storage of nasopharyngeal swabs at ULT in STGG[8]. These conditions may be difficult to achieve at field sites or during sample transport, particularly in isolated communities or other challenging settings. Based on our study threshold, -20°C in STGG was deemed the only suitable alternative for pneumococcal detection via culture when stored for up to 28 days, however density was greatly affected. Previous studies have recommended pneumococcal specimens not to be stored at -20°C for > 4 months[11] Our findings showed inconsistent recovery across serotypes tested, and support findings from another study that storage at 4°C for > 5 days was unacceptable for preservation of viable pneumococci[17]. We also found SDPs to be unsuitable for pneumococcal carriage studies, consistent with findings that delayed culture and transport at ambient temperatures may lead to underestimations of pneumococcal prevalence[13,18]. We noted that all the alternative storage conditions had substantive declines in pneumococcal density using culture-based methods and are therefore not appropriate to use in carriage studies where density is an important endpoint. However, these methods may still be useful in carriage studies employing fully molecular approaches, where expected storage or transit times are limited, or for shipping pure isolates if using SDPs.

There are important limitations of this study. We utilised laboratory prepared samples testing a limited isolate collection, and where each mock specimen only contained a single serotype of pneumococcus. Future studies are needed to determine if the results are representative of the serotype (and all

pneumococci) more generally, for multiple serotype carriage, and mixed microbial samples. Our study has several strengths, particularly in that it provides solutions for real-world scenario testing. Samples were also designed to mimic biologically relevant densities[19], with a focus on the lower end of bacterial densities where viability affects are more likely to be noticed.

Pneumococcal carriage is not limited to populations or regions with good access and laboratory infrastructure. The feasibility of storage and transport conditions should be considered in relation to the study outcomes of interest, as improper storage of specimens may lead to underestimations of pneumococcal prevalence and density, especially when culture-based methods are used. These findings may allow future carriage studies to reach underrepresented or previously unrepresented communities or regions. For example, we were able to successfully employ short-term – 20°C storage of samples in our studies in an internally displaced person's camp in Somaliland [9]. It also highlights that where viable pneumococci are not required, molecular methods could be utilised to provide useful information despite logistical challenges.

Conclusion

Conducting pneumococcal carriage studies in accordance with WHO guidelines should still remain the priority in settings that allow for it. However, in the absence of ULT storage, or when faced with other logistical challenges, -20°C storage of samples in STGG presents a minimal loss alternative for culture based pneumococcal detection up to 28 days. We also demonstrate the utility, and benefit, of molecular methods where temperature-controlled transport or storage is challenging or interrupted.

Abbreviations

CFU: Colony forming units

CI: Confidence intervals

GE: Genome equivalents

HBA: Horse blood agar

LMICs: Low- and middle-income countries

qPCR: Quantitative real-time PCR

SD: Standard deviation

SDP: Silica desiccant packets

STGG: Skim milk, tryptone, glucose and glycerol

ULT: Ultra-low temperatures

WHO: World Health Organization

Declarations

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Declaration

Not applicable

Competing Interests

AWH, CP, BO, MLN, FR, OT, HPT, TVN, BT, AIH, SAI, SF, KVZ, FC and EN none. CVM, EKM, TM, and CS were investigators on a clinical research collaboration with Pfizer on PCV vaccination in Mongolia from which two isolates used in this study are derived. Salary support was received through the institutions. CVM has received honoraria [Pfizer and MSD] for expert advisory meetings unrelated to this study.

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Author Contributions

AWH, CP, BDO, EN and CS conceived the study and designed the experiments. AWH and CP conducted the laboratory work. MLN provided statistical assistance. AWH conducted the data analysis. AWH, CP, EN and CS drafted the manuscript. EN and CS oversaw the study. All authors read and approved the final manuscript.

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Figures

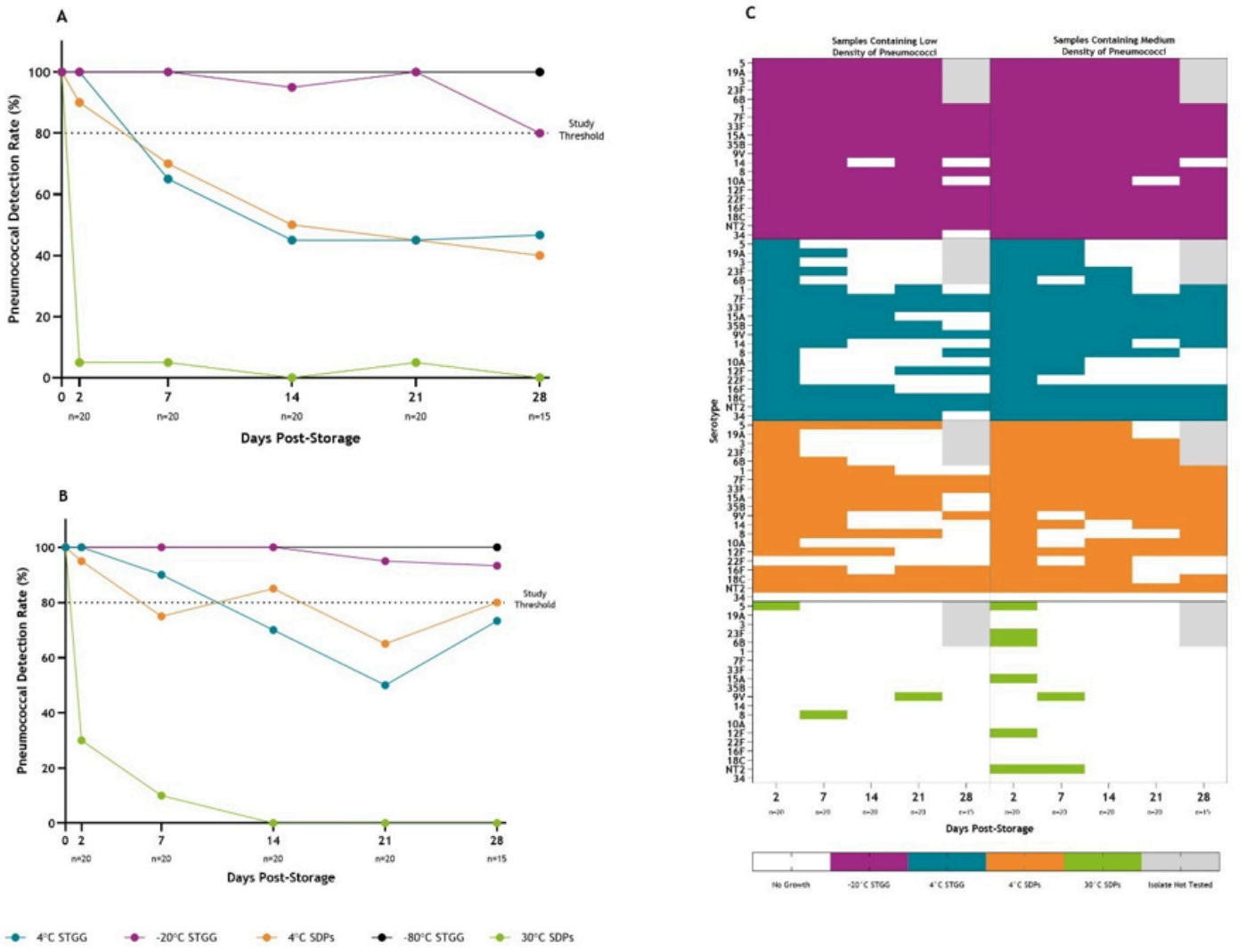

Figure 1

Detection rate of viable pneumococci at baseline, 2, 7, 14, 21 and 28 days post-storage for samples inoculated with a A) low bacterial load (10^2 - 10^3 CFU/ml) of pneumococcus and B) medium bacterial load (10^4 - 10^5 CFU/ml) of pneumococcus. Twenty isolates, each representing a different serotype, were stored for each bacterial load at 4°C in STGG (blue), -20°C in STGG (purple), 4°C in silica desiccant packets (SDPs; orange), 30°C in SDPs (green) and -80°C in STGG (black); (n = 790 samples total (Supplementary Figure 1)). Closed circles denote the median pneumococcal detection rate of all the samples tested at that timepoint. Dotted line indicates the study threshold (80%). C) Heatmap depicting recovery of pneumococci (≥1 colony) at 2, 7, 14, 21 and 28 days post-storage. Results are presented for individual serotypes and bacterial loads, for each storage condition tested. Coloured squares denote viable culture (-20°C STGG (purple), 4°C STGG (blue), 4°C SDPs (orange), 30°C SDPs (green)), white squares represent no growth, and grey squares indicate testing was not conducted.

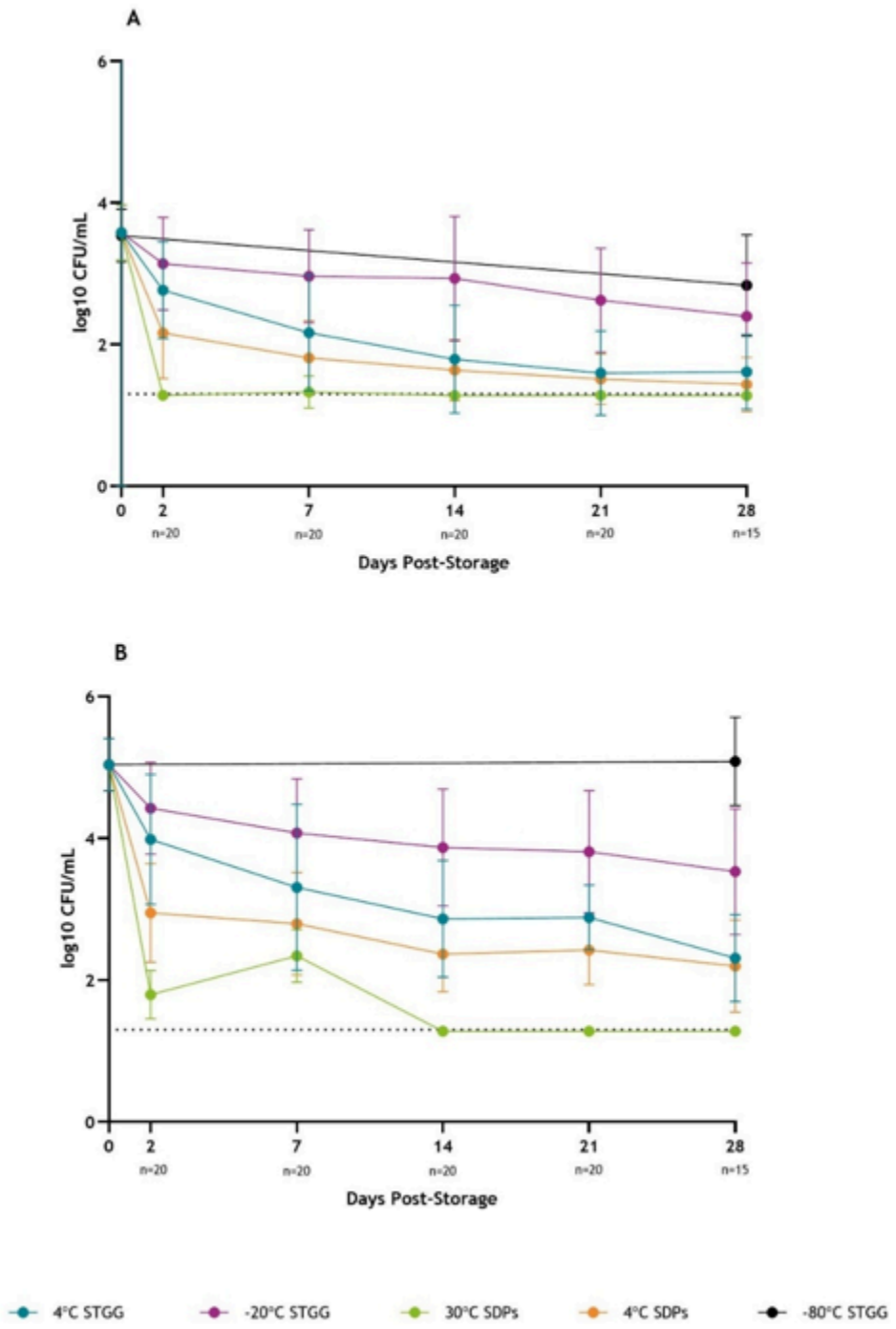

Figure 2

Pneumococcal density (CFU/ml) enumerated by viable count at baseline, 2, 7, 14, 21 and 28 days post-storage for each storage condition (-20°C STGG (purple), 4°C STGG (blue), 4°C SDPs (orange), 30°C SDPs (green) and -80°C in STGG (black) for samples spiked with A) low bacterial loads (10^{2} - 10^{3} CFU/ml) and B) medium bacterial loads (10^{4} - 10^{5} CFU/ml) of pneumococcus. Values have been log transformed. Closed circles denote the median CFU/ml calculated for each condition at that timepoint. Dotted line indicates the limit of detection (n = 20 CFU/ml). Error bars indicate standard deviation (SD).

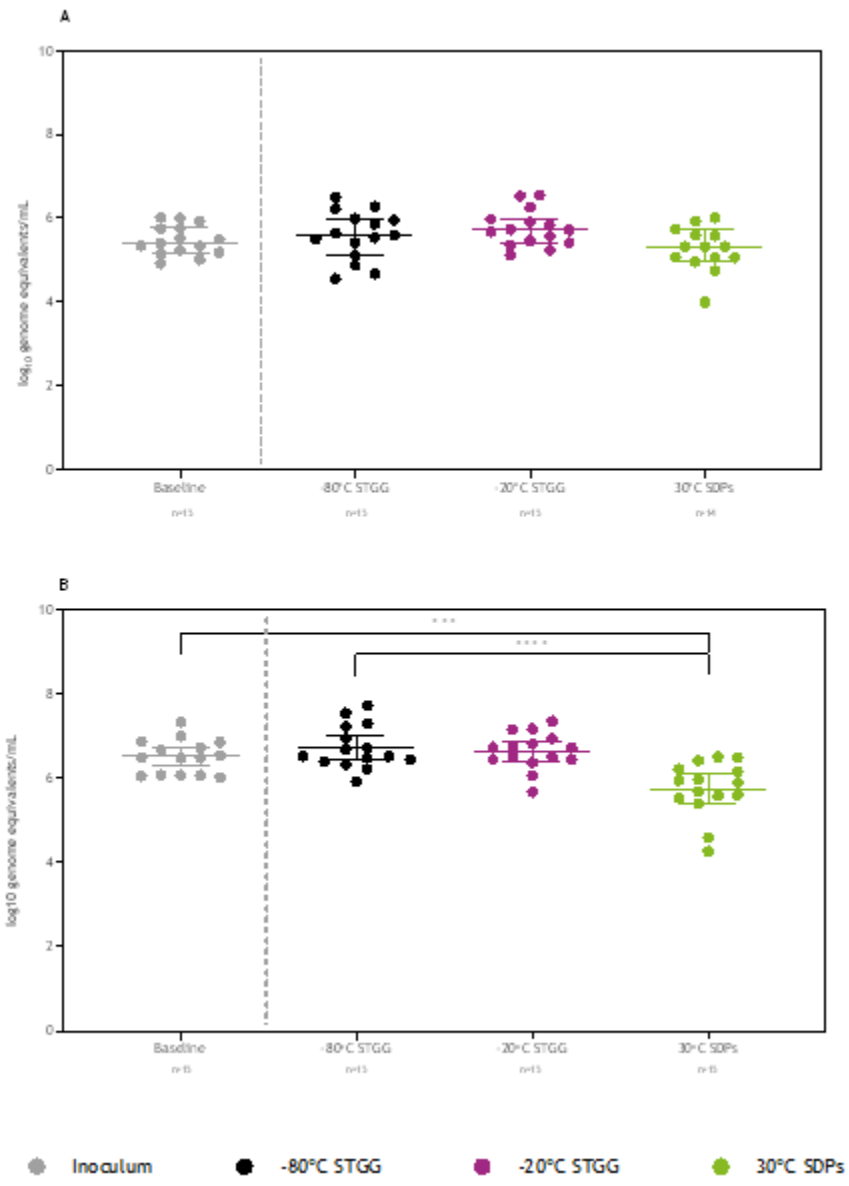

Figure 3

Pneumococcal density (log_{10} genome equivalents (GE)/ml) was detected by *lytA* qPCR at baseline (inoculum, grey), and 28 days following storage at -80°C in STGG (gold standard, black), -20°C in STGG (purple) and 30°C in silica desiccant packets (SDPs, green) for samples spiked with a) low bacterial loads (10^2 - 10^3 CFU/ml) and B) medium bacterial loads (10^4 - 10^5 CFU/ml) of pneumococcus. Values have been log transformed. Closed circles represent the density (log_{10} GE/ml) calculated for each sample. Lines depict the mean and error bars indicate the standard deviation (SD). Asterisks denote the level of statistical significance in a comparison between groups.

Supplementary Files

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