Mechanically ventilated patients shed high titre live SARS-CoV2 for extended periods from both the upper and lower respiratory tract

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Summary: Patients on intensive therapy infected with SARS-CoV-2 tend to be prolonged shedders, excreting virus for far beyond the time periods specified in current guidelines, and live virus titres can be extremely high in both the upper and lower respiratory tracts.

ABSTRACT

Background

SARS-CoV-2 infection can lead to severe acute respiratory distress syndrome needing intensive care admission and may lead to death. As a virus that transmits by respiratory droplets and aerosols, determining the duration of viable virus shedding from the respiratory tract is critical for patient prognosis, and informs infection control measures both within healthcare settings and the public domain.

Methods

We prospectively examined upper and lower airway respiratory secretions for both viral RNA and infectious virions in mechanically ventilated patients admitted to the intensive care unit of the University Hospital of Wales. Samples were taken from the oral cavity (saliva), oropharynx (sub-glottic aspirate), or lower respiratory tract (non-directed bronchoalveolar lavage (NBL) or bronchoalveolar lavage (BAL)) and analyzed by both qPCR and plaque assay.

Results

117 samples were obtained from 25 patients. qPCR showed extremely high rates of positivity across all sample types, however live virus was far more common in saliva (68%) than in BAL/NBAL (32%). Average titres of live virus were higher in subglottic aspirates ($4.5x10^7$) than in saliva ($2.2x10^6$) or BAL/NBAL ($8.5x10^6$) and reached $>10^8$ PFU/ml in some samples. The longest duration of shedding was 98 days, while most patients (14/25) shed live virus for 20 days or longer.

Conclusions

Intensive care unit patients infected with SARS-CoV-2 can shed high titres of virus both in the upper and lower respiratory tract and tend to be prolonged shedders. This information is important for

decision making around cohorting patients, de-escalation of PPE, and undertaking potential aerosol generating procedures.

Keywords: SARS-CoV-2, Plaque Assay, qPCR, Intensive Care Unit, coronavirus disease 2019, viral load

INTRODUCTION

The COVID-19 pandemic has resulted in a global human death toll of 5.36 million (as of 21/12/21) [1]. Early symptoms include a dry cough, exertional shortness of breath, fatigue, lethargy, diarrhea and high-grade fever [2], and in 10-15% of cases, this can progress to severe pneumonia needing hospitalization. In 1-2% of cases the disease can lead to severe acute respiratory distress syndrome (ARDS) needing ICU admission and may lead to death [3]. As a virus that transmits by respiratory droplets and aerosols, determining the duration of viable virus shedding from the respiratory tract is critical for patient prognosis, and informs infection control measures both within healthcare settings and the public domain [4]. Whilst symptoms may persist for weeks or even months post infection, shedding of infectious viral particles almost never occurs beyond 10 days of symptom onset, even in hospitalized patients [5]. In a meta-analysis including >5000 SARS-CoV-2 infected individuals, viral RNA was detectable up to 83 days in the upper respiratory tract, but no study detected live virus beyond day 9 of illness [5]. However, this analysis was performed prior to the introduction of immunosuppressive agents as standard of care for individuals hospitalised with severe respiratory complications of COVID-19 [6]. Given emerging evidence that infectious virions can be recovered from individuals with acquired and inherited forms of immunodeficiency months after symptom onset [7-10], we investigated whether adults requiring admission to ICU, who are subject to both infection-mediated immune dysregulation [11] and iatrogenic immunosuppression [6], exhibited prolonged viral shedding. Furthermore, no study has investigated whether the 9 day 'cut-off' for live virus isolation applies to the lower respiratory tract or airways.

We examined upper and lower airway respiratory secretions in mechanically ventilated COVID-19 patients admitted to the ICU of the University Hospital of Wales for titres of infectious SARS-CoV-2, and compared this to qPCR. We show that infectious viral particles are readily recoverable from saliva and that these patients can secrete extremely high levels of live SARS-CoV-2 from multiple

sites in the respiratory tract well beyond the 20-day isolation period currently recommended by the CDC for patients with severe COVID-19 [12], and thus represent a nosocomial reservoir of infection.

METHODS

Sample collection

Saliva was collected using Neutral Salivettes® (SARSTEDT, Germany) which were placed against the buccal mucosa for two minutes and then spun (2000xg) to collect supernatant, or washed with DMEM if no supernatant was present. Subglottic endotracheal tubes are used in many ICUs as they reduce ventilator associated pneumonia. Subglottic aspirates represent an accumulation of oropharyngeal secretions which accumulate above the endotracheal cuff. Broncholaveolar lavage was undertaken using a disposable Ambu® aScope™ 4 and Broncho Sampler Set (Ambu UK) with lavage of up to 80ml of sterile saline, alternatively a non-directed bronchoalveolar lavage was performed by inserting a suction catheter into the lung until resistance was met and 20ml of sterile saline inserted and slowly withdrawn. All patients received evidence-based treatment as per published health board or ICU directorate guidelines. Samples were transferred to the BSL3 laboratory and processed within 4h. Baseline characteristics and treatments are in table 1.

Trial design

Sample collection (20/10/20 – 8/12/20) was undertaken as a service evaluation to see if virus could be measured in respiratory tract samples as an alternative to RT-qPCR. From 01/02/21 – 31/03/21, participants were enrolled in ENLIST (REC Reference 20/YH/0309) and consent taken from a relative or legal representative. Sampling was prospective, and weekly unless clinically indicated otherwise. Inclusion criteria were: 1) ≥18 years, 2) Clinical presentation of COVID-19, 3) PCR positive for SARS-CoV-2, 4) Admitted to ICU requiring Invasive mechanical ventilation. Exclusion criteria were <18 years.

Plaque assays

Cells were grown in DMEM containing 10 % (v/v) FCS and incubated at 37°C in 5% CO_2. Plaque assays utilised VeroE6 expressing ACE2 and TMPRSS2 to enhance virus entry [13]. Serial dilutions of sample were applied to cells for 1 h at 37°C with rocking. Cells were overlaid with DMEM containing 2 % FCS, 1.2 % Avicel®, 50 ug/mL Gentamycin (Fisher Scientific, UK) and 2.5 ug/mL Amphotericin-B (Sigma Aldrich, UK). After 72 h, overlay was removed, the monolayer washed, fixed with 100% methanol, stained with 25% (v/v) methanol and 0.5 % (w/v) Crystal Violet, then washed, and plaques enumerated.

RNA extraction

Sample (100uL) was incubated with 10 uL of Proteinase K (Qiagen UK) for 15 minutes at room temperature, then incubated at 70°C for 15 minutes to inactivate enzyme. 10uL of RQ1 DNase buffer (Promega, UK) and 10uL of RQ1 DNase (Promega, UK) was added, then incubated at 37°C for 30 minutes. RNA was extracted using the QIAmp Viral RNA Minikit (Qiagen, UK), and eluted in 60uL.

qPCR

RT-qPCR for SARS-CoV-2 was carried out using E-gene targeting primers and probe: ACAGGTACGTTAATAGTTAATAGCGT, ATATTGCAGCAGTACGCACACA, FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ. Copy number was quantified using a control plasmid (pEX-A128-nCoV_E_Sarbeco, Eurofins Genomics, Germany). RNA quality was assessed by RNAse P detection [14] using primers and probe; AGATTTGGACCTGCGAGCG, GAGCGGCTGTCTCCACAAGT, FAM-TTCTGACCTGAAGGCTCTGCGCG-BBQ. Reactions were carried out in 20uL volumes containing; 4.4uL QuantiTect Virus Mastermix (Qiagen, UK), 0.2uL QuantiTect Virus RT Mix, 0.4uM forward primer, 0.4uM reverse primer, 0.2uM probe, 1uL RNA, 0.5uL non-acetylated BSA (2mg/mL, Sigma-Aldrich, UK). RT-qPCR was conducted on a QuantStudio 3 (ThermoFisher Scientific, UK) with the

following cycle conditions; 50°C for 20 minutes, 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 58°C for 45 seconds.

SARS-CoV2 variant identification

Variants were analysed by sequencing a portion of the Spike gene using the following primers; GTGTTAATCTTACAACCAGAACTCAATTAC, CACAGACTTTAATAACAACATTAGTAGCG. RT-PCR conditions were as above, except that the annealing temperature was 55°C. Sanger sequencing used the same primers (Eurofins Genomics, Germany).

Statistics

Distribution of continuous variables was analysed for normality using Shapiro-Wilk test. Differences between sample types were analysed using analysis of variance and Kruskal-Wallis test followed by Dunn's multiple comparison test. Spearman's rank correlation assessed the relationship between qPCR and plaque assay results. Analyses were carried out in GraphPad Prism 6 (GraphPad Software).

RESULTS

117 samples (44 saliva, 32 subglottic, and 41 BAL) were obtained from 25 adults admitted to the ICU at the University Hospital for Wales, a tertiary referral center. All patients had a diagnosis of SARS-CoV-2 based on nasopharyngeal swab, and none had received a SARS-CoV-2 vaccine. The median age was 59 years (range 37 to 76), with a male bias (16/25, 64%). All were sedated and mechanically ventilated throughout the study, with the majority immunosuppressed as a result of treatment with Dexamethasone (92%) and Tocilizumab (40%) as part of their COVID-19 evidence-based therapy (10,11).

To determine whether levels of virus shedding varied across sites within the respiratory tract, samples of NBAL/BAL, subglottic aspirate, and saliva, were taken and assessed for RNA genome levels, and live virus titres (figure 1). It was not always possible to collect all sample types at each

timepoint, especially where it was felt that NBAL/BAL might further compromise the patient's respiratory capacity. To avoid bias from variable sampling times, data was analyzed across all samples (Figure 1A), and having excluded timepoints with incomplete data (Figure 1B). Consistent with diagnosis at admission, qPCR showed extremely high rates of positivity across all sample types (93 – 97%). In contrast, detection rates for live virus varied between sample types. Most saliva samples (30/44; 68%) contained live virus (figures 1 and 2), however this was not the case in subglottic aspirates and BAL/NBAL samples. Nevertheless, infectious virions were still detected in 14/32 (44%) subglottic aspirate samples and 13/41 (32%) BAL samples (figures 1 and 2).

Live virus titres varied from the limit of detection (10 PFU/ml) to extremely high (>10^8 PFU/ml), and were significantly higher in saliva than BAL. Across all samples, average titres reflected the chances of recovering live virus from any sample, with saliva containing the highest (1 x 10^3 PFU/mL), while subglottic aspirates were slightly lower (2.5 x 10^2 PFU/mL), and BAL lower still 1 x 10^1 PFU/mL (Figure 1). In contrast, when samples from which virus could not be isolated were excluded, subglottic aspirates contained higher titres of live virus (4.5 x 10^7 PFU/mL) than either saliva (2.2 x 10^6 PFU/mL) or BAL/NBAL (8.5 x 10^6 PFU/mL). This latter result was also reflected in genome copy numbers, which were notably higher in subglottic aspirates than saliva.

Previous studies have demonstrated a correlation between qPCR Ct value and the chances of recovering live virus from oral swabs, with isolation of live virus becoming more infrequent as Ct values increase. In accordance with this, qPCR was clearly more sensitive than virus isolation across all sample types. However, when virus and genome titres were compared (figure 3), we observed a moderate, significant correlation for saliva but not for subglottic aspirates or BAL/NBAL. In saliva, samples lacking live virus all had genome titres below 10^4 copies/ml, suggesting a 'cut-off' for detection of infectious virus. However, amongst samples containing live virus, genome titres were as low as 10^2 copies/ml. The correlation was even weaker in BAL and subglottic aspirates, where we

failed to isolate live virus from samples containing RNA levels as high as 10^9 genomes/ml, but successfully isolated virus from samples with genome titres of 10^3 copies/ml.

In previous data live virus was rarely detected beyond 10 days after symptom onset from oro- or nasopharyngeal swab samples, even amongst hospitalized patients [5]. The situation was markedly different in our cohort, where 16 of the 25 patients (64%) shed viable virus for longer than 10 days (figure 4, supplementary data 1). The longest duration of shedding was 98 days, while the majority of patients (14/25 (56%)) shed virus for 20 days or longer. Saliva and subglottic aspirate tended to remain positive for longer than BAL, in accordance with our previous observation that BAL was the sample least likely to contain viable virus.

While this study was underway, the Alpha variant of concern began to spread. We therefore sequenced the Spike gene to determine whether the isolation of viable virus differed based on strain. No clear differences were seen in the longevity of virus isolation. Furthermore, no correlation was observed between viral load and patient outcome.

When viable viral and gene copy loads from each patient were compared longitudinally, the highest viral titres across all patients were recorded from subglottic aspiration samples. However overall, saliva provided a better indication of infection; whenever live virus was isolated from any sample at any timepoint, saliva at that timepoint always contained live virus. In contrast, by qPCR, sample type was largely irrelevant for determining positivity. There were however differences in viral load by qPCR, with subglottic aspirates often containing higher titres than saliva.

DISCUSSION

Current NHS guidance states isolation precautions can be discontinued in individuals with SARS-CoV-2 infection 10 days after symptom onset [15], whilst the CDC recommends extending this for up to 20 days after symptom onset in those with severe illness [12]. Our study clearly demonstrates that ICU patients frequently excrete high titres of infectious SARS-CoV2 for periods far exceeding these

recommendations. Viral titres from saliva, subglottic aspirate, and BAL/NBAL can reach >10^7 PFU/ml in some patients. This was not variant specific as individual patients infected by either Alpha, or earlier variants, shed these high titers of infective virus. Our study also highlights the inadequacy of qPCR in determining the point during the infection course when an intubated patient ceases to present an infection risk to hospital staff; this was particularly true for airway and lower respiratory tract samples, where PCR positivity was poor at predicting live virus. Furthermore, in contrast to studies using oral swabs in hospitalized patients [16], Ct values of ICU patients were not a good predictor for the presence of live virus, a problem that has been highlighted in previous studies investigating discrepancies between RT-PCR and symptomatic infection [17-19].

This adds to an increasing body of evidence relating to the extended duration of live virus shedding from ventilated COVID-19 patients. Van Kampen *et al* grouped patients from wards and ICU and showed that live virus can be isolated from the sputum and upper respiratory tract samples of severe or critical COVID patients for >10 days, however this was only in a small proportion (10%) of samples, with the median duration of shedding being 8 days from symptom onset [20]. Furthermore, whether the samples containing live virus were sputum or upper respiratory samples was not defined, whether long-term shedding correlated with mechanical ventilation was not examined, and only 1 patient excreted live virus for up to 20 days. A second study examined patients in ICU, of whom 72% were mechanically ventilated [21]. They found that patients excreted virus for a median of 13 days from the upper respiratory tract, and only two remained positive up to 20 days. We observed much longer durations of shedding, with most patients excreting virus >20 days, up to a maximum of 98 days. We also isolated live virus from a much greater proportion of patients (87%) compared to previous studies (17.8% and 7% respectively). This may reflect the clinical characteristics of our cohort; all were mechanically ventilated and the majority had failed steroid therapy on the wards (whereas earlier cohorts were steroid naïve) which is reflected in their high mortality (64%). It may also reflect our use of saliva – sample type was not specified in previous studies [20, 21], however nasopharyngeal swabs are common. These capture a small sample

quantity and dilute it further in transport medium. In addition, previous studies did not isolate virus on cells expressing human ACE2 and TMPRSS2; expression of these proteins represents a more biologically relevant target cell and significantly enhances virus detection [13]. This highlights the need to use sensitive methodologies and repeat sampling before concluding that patients are not secreting live virus. Our data also extends these previous studies by correlating upper and lower respiratory tract samples and demonstrating that live virus shedding also occurs from the lower respiratory tract for extended durations.

Only one other study has titrated live virus from clinical samples. Differences in sample and cell type can alter viral titer readouts, nevertheless, this study demonstrated titres of $5x10^6$ and $4x10^6$ PFU/ml in nasopharyngeal swabs from two patients [22]. Thus, despite their prolonged shedding, titres in ICU patients may not be substantially different to those in milder disease. Mouth swabs are commonly used to diagnose SARS-CoV-2 infection and may be interpreted as a surrogate for shedding of live virus. However, the respiratory droplets that transmit virus have been assumed to arise from both the upper and lower respiratory tract. Previous studies have compared genome loads in BAL compared to mouth swabs using qPCR [23-24]. In agreement with these studies, we find that PCR is largely concordant between upper and lower respiratory tract samples, thus BAL/NBAL do not offer advantages over the more practical upper respiratory samples for diagnosis. However, in contrast to viral genome, live virus was much more common in saliva than BAL/ NBAL, suggesting that the upper respiratory tract is more likely to be a source of infectious virus than the lower. This is consistent with previous reports that there is independent replication of virus in the upper and lower airways [25]. However, it may also reflect the chances of virus being inactivated in a sample containing high levels of mucus and other proteolytic enzymes, and the volume of fluid used to lavage the lungs' parenchyma. Titres in BAL/NBAL may therefore under-estimate the true situation. Nevertheless, it is clear that cell free live virus is capable of reaching extremely high titres in the lungs, and is a potential source of transmissible virus in a proportion of patients. This discordance between titres of infectious virus and viral genomes in BAL/NBAL highlights the advantage of

measuring infectious viral load directly by plaque assay. Commonly implemented indirect viral load measurement methods, such as measuring gene copies and subsequently confirming the sample to contain infectious virions by observing CPE on cultured cells [20-21, 25-27], would have resulted in over and under estimation of infectious viral load in numerous samples.

In a proportion of samples, genome titres were lower than live virus titres. This likely reflects the difficulty of extracting RNA from a highly proteolytic sample, and the need to process the sample to exclude carry-through inhibitors – problems which are reduced in nasopharyngeal swabs that most studies use. All qPCR reactions were controlled by RNaseP, to ensure that inhibitors did not affect results, and this is reflected in the fact that nearly all samples were positive for viral RNA. Nevertheless, the higher processing requirements, and the lability of RNA, may result in the genome copy number being an under-representation of the in vivo situation. Despite this, the genome load in our cohort was similar to those previously reported from oropharyngeal [16] and saliva [28] samples in hospitalized patients.

Our study demonstrates that qPCR is not a robust indicator of viable viral shedding in critically ill patients, irrespective of sample type. Patients on ICU infected with SARS-CoV-2 tend to be prolonged shedders, excreting virus for far beyond the time periods specified in current guidelines, and virus titres can be extremely high in both the upper and lower respiratory tracts. This may be a consequence of infection induced immunosuppression and/or the use of steroids and/or IL-6 blockade to limit tissue damage. This information is important for decision making around cohorting patients, de-escalation of PPE, and undertaking potential aerosol generating procedures, particularly given the threat of new variants such as Omicron that have higher transmission rates and greater vaccine escape potential. It also supports the continued use of oral antiseptics in these patients; antiseptics such as chlorhexidine are used routinely to reduce ventilator-associated pneumonia [29]. These may also have a role to play in minimising nosocomial transmission, although formulations containing surfactants are likely to be most effective [30]. Larger multi-centre cohorts are now

needed to determine the clinical features that correlate with long-term shedding (e.g. age, humoral and cellular immune responses, specific treatments), and to assess whether the use of monoclonal antibody therapies, vaccination, and antivirals can reduce persistent shedding. Our study also highlights the need for more robust, practical assays for the determination of viable viral shedding in healthcare settings.

NOTES

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CONFLICT OF INTEREST

The authors declare no conflict of interest. MPW reports being co-applicant on grant application to institution from UKRI-BBSRC Covid-19 Agile Response Call - Mapping the lipid envelope composition of SARS-CoV-2 for reducing transmission, thrombosis and inflammation – co-applicant £597,540 and payment for lectures from Gilead and Fisher & Paykel. MJP reports payments to institution from Association of Clinical Pathologists (ACP) UK Career Development Award outside of the submitted work.

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FIGURE LEGENDS

Figure 1: Comparisons of viable viral and gene copy load from saliva, subglottic aspirate, and bronchoalveolar lavage as determined by plaque and qPCR assays for a. all samples processed in this study b. subgroup wherein sampling timepoints that were incomplete (did not contain all three sample types) were omitted. Lines represent the geometric means, *=P<0.05, **=P<0.01, ***=P<0.001 and NS=not significant (Kruskal-Wallis test with Dunn's multiple comparison test).

Figure 2: Percentage of all saliva, subglottic aspirate, and bronchoalveolar samples that were found to be positive by qPCR and plaque assays for a. all samples processed in this study b. subgroup wherein sampling timepoints that were incomplete (did not contain all three sample types) were omitted.

Figure 3: Correlation between viral load as determined by plaque assay (PFU/mL) and gene copies/mL as determined by qPCR assay. Comparisons were made between the saliva, subglottic aspirations, and BAL/NBAL sample types. The dashed line represents equal titres of the gene copy and viable viral loads (Spearman's Rank).

Figure 4: Longitudinal analyses of patient viral and gene copy loads as determined by plaque and qPCR assays, respectively. A cross inside a circle above the patient number indicates a fatal outcome for the patient. Asterisks above a patient block indicate the patient was infected with the Alpha variant (B.1.1.7).

Table 1. Baseline Characteristics and Treatments of Patients

Variable	
Age in years, Median (IQR)	59 (50 - 68)
Female sex, Number (%)	9 (36%)
In-hospital mortality, Number (%)	16 (64%)
Received Dexamethasone treatment, Number (%)	23 (92%)
Received Remdesivir treatment, Number (%)	15 (60%)
Received Tocilizumab treatment, Number (%)	10 (40%)

Figure 1

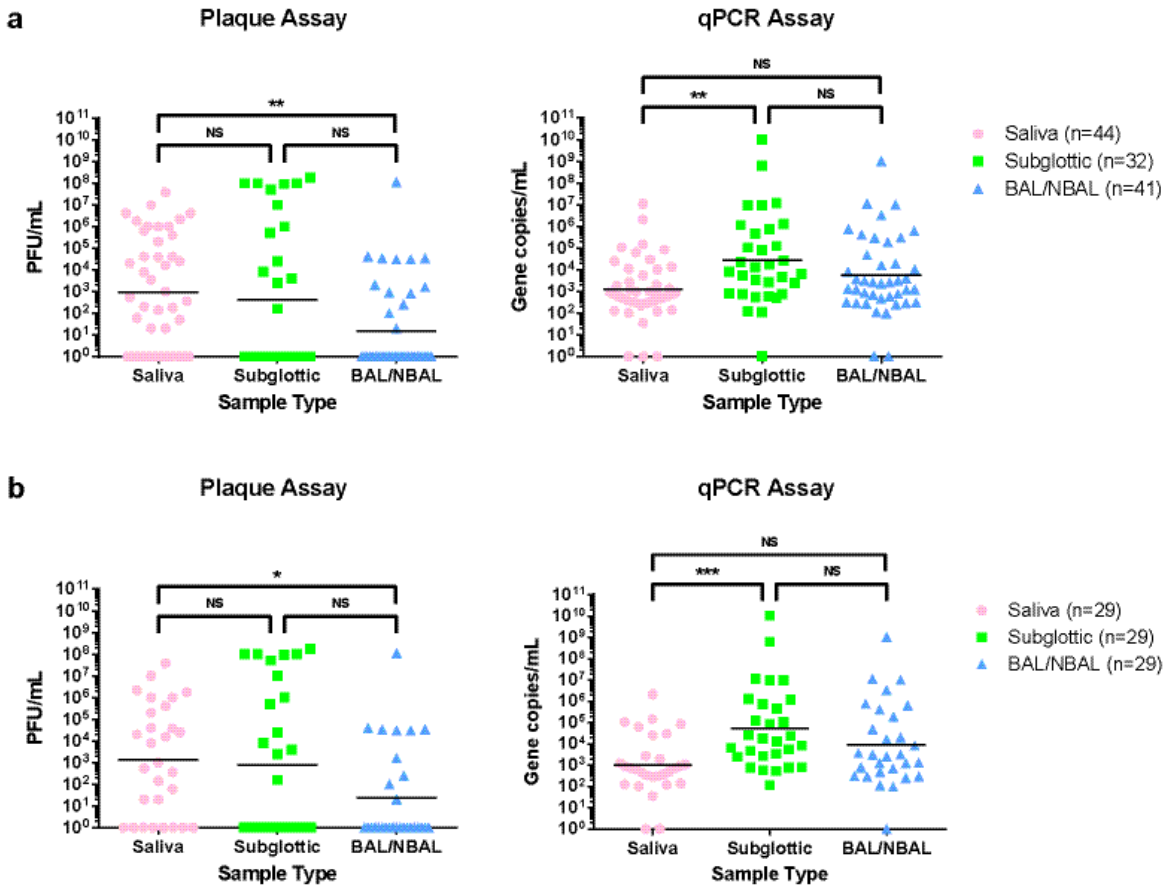

Figure 2

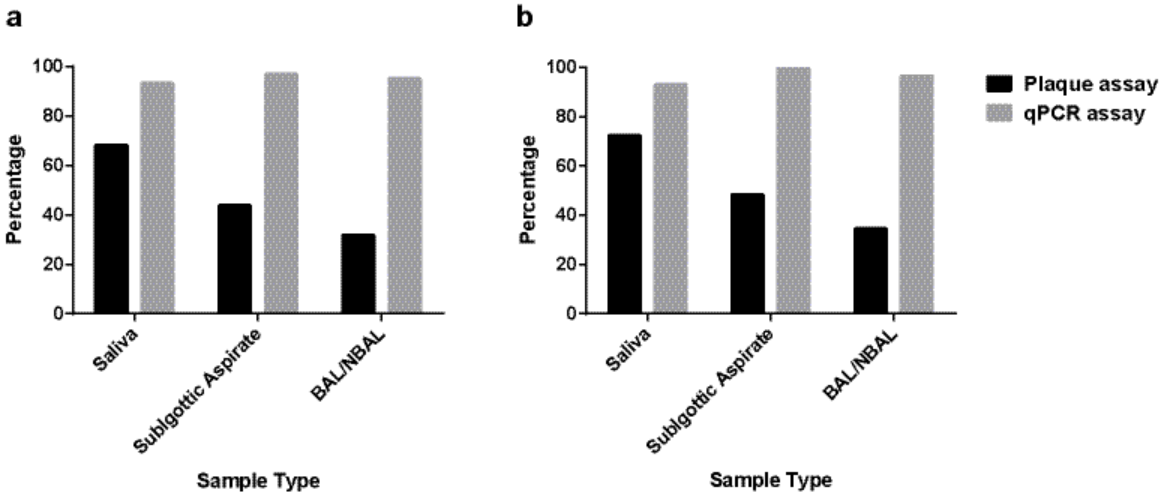

Figure 3

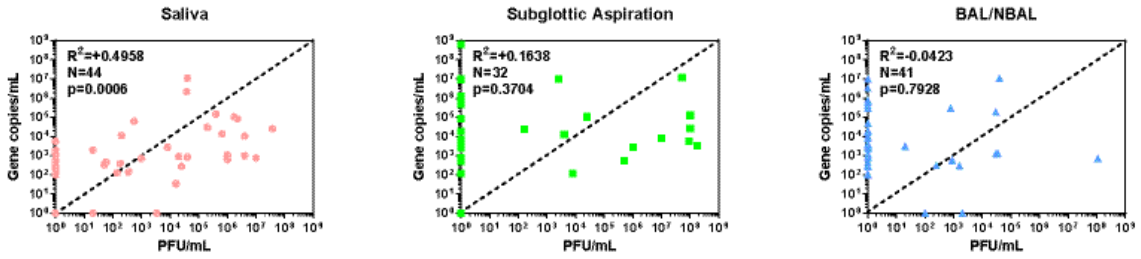

Figure 4