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Cellular DNA quantification in respiratory samples for the normalization of viral load: a real need?

Antonio Piralla[a,1], Federica Giardina[a,1], Francesca Rovida[a], Giulia Campanini[a], Fausto Baldanti[a,b,*]

[a] Molecular Virology Unit, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[b] Department of Clinical-Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy

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ABSTRACT

Background: Respiratory tract infections have an enormous social economic impact, with high incidence of hospitalization and high costs. Adequate specimen collection is the first crucial step for the correct diagnosis of viral respiratory infections.

Objectives: The present retrospective study aimed: i) to verify the cell yield obtained from sampling the nasal respiratory tract using mid-turbinate flocked swabs; ii) to evaluate the normalization of viral load, based on cell number; and iii) to compare the kinetics of viral infection obtained with normalized *vs* non-normalized viral load.

Study design: The number of cells were quantified by real-time PCR in residual extract of nasal swabs tested for respiratory viruses detection and stored at −80 °C in a universal transport medium (UTM™).

Results: A total of 513 virus-positive and 226 virus-negative samples were analyzed. Overall, a median of 4.42 $\log_{10}$ β2-microgolubin DNA copy number/ml of UTM™ (range 1.17–7.26) was detected. A significantly higher number of cells was observed in virus-positive as compared to virus-negative samples (4.75 *vs* 3.76; $p < 0.001$). Viral loads expressed as $\log_{10}$ RNA copies/ml of UTM™ and $\log_{10}$ RNA copies/median number of cells were compared in virus-positive samples and a strict correlation ($r = 0.89$, $p < 0.001$) and agreement ($R2 = 0.82$) were observed. In addition, infection kinetics were compared using the two methods with a follow-up series of eight episodes of viral infection and the mean difference was -0.57 $\log_{10}$ (range −1.99 to 0.40).

Conclusions: The normalization of viral load using cellular load compliments the validation of real-time PCR results in the diagnosis of respiratory viruses but is not strictly needed.

1. Background

Respiratory tract infections (RTI) have an enormous social and economic impact, with a high incidence of hospitalization and high public health care costs [1]. Because of similar clinical symptoms and simultaneous circulation of several different viruses, their etiology is often difficult to determine. Adequate specimen collection is the first crucial step for the correct diagnosis of influenza and other respiratory infections. Dilution correction in nasopharyngeal aspirates might improve the detection of respiratory infections [2]. Many studies have confirmed that mid-turbinate flocked swabs are less invasive than other specimen collection types (nasopharyngeal swabs, aspirates and washes), have good sensitivity in the detection of respiratory viruses and are therefore a good alternative for specimen collection [3–6]. Moreover, these mid-turbinate flocked nasal swabs are suitable for self-collection at home (either in adult patients or in children by their parents). Fast- and high-throughput molecular workflows require sample matrices that are suitable for automation. Respiratory swab specimens are better suited for this purpose compared to the more viscous nasopharyngeal aspirates. Universal Transport Medium (UTM™) is a room temperature stable viral transport medium for the collection, transport, maintenance and long term frozen storage of viruses and other pathogens such as Chlamydia, Mycoplasma and Ureaplasma.

* Corresponding author at: Molecular Virology Unit, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, via Taramelli 5, 27100 Pavia, Italy.
E-mail addresses: f.baldanti@smatteo.pv.it, fausto.baldanti@unipv.it (F. Baldanti).
[1] These authors contributed equally to this work.

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2. Objectives

The present retrospective study aimed: i) to verify the cell yield obtained from sampling the nasal respiratory tract using mid-turbinate flocked swabs, subsequently stored in UTM™; ii) to evaluate the normalization of viral load, based on cell number; and iii) to compare the kinetics of respiratory viral infection obtained with normalized *vs* non-normalized viral load.

3. Study design

3.1. Study design and samples

For the present study, a total of 739 residual UTM™ extracts stored at −80 °C and collected from December 2013 through April 2014 at the Molecular Virology Unit of the Fondazione IRCCS Policlinico San Matteo were included. A series of single or follow-up samples were also analyzed.

As part of standard diagnostic procedures, UTM™ extracts were tested with a panel of laboratory-developed real-time RT-PCR or real-time PCR to detect and quantify the following viruses: influenza virus A, human rhinoviruses (HRV), respiratory syncytial virus (RSV) types A and B, and human coronaviruses (hCoV) − OC43, -229E, -NL63, and -HKU1, as previously described [7,8].

Viral DNA/RNA was extracted from 500 µl (1:2 to ml) of mid-turbinate flocked nasal swabs (FLOQSwabs®, Copan Italia SpA, Brescia, Italy) stored in UTM™ (Copan Italia SpA, Brescia, Italy) on the automated extraction system NucliSENS® easyMAG™ (BioMerieux, Lyon, France). Elution volume was 55 µl, 5 µl (1:11) of which were used for respiratory viruses detection described above [7,8]. Viral RNA load was expressed per ml of UTM™ (copies or $\log_{10}$ copies/ml of UTM™) according to the extraction procedure dilution factor and calculated as follows: [(RNA copies number per reaction) × 22].

3.2. Cells quantification

In order to assess the sample adequacy, the number of respiratory epithelial cells was counted by quantifying the DNA of the housekeeping gene β2-microglobulin by real-time PCR, as previously described [9]. The number of cells was reported as β2-microgloubin DNA copy number/ml of UTM™ [10].

3.3. Viral load normalization

The normalized viral RNA load value was expressed as the number of viral RNA copies (copies or $\log_{10}$ copies) per median number of cells recovered in positive samples (4.65 $\log_{10}$ β2-microgolubin DNA copy number/ml of UTM™), and calculated as follows:

$$\text{normalized viral RNA load (ml of UTM™)} = \frac{[\text{RNA copies reaction} * 45282]}{\beta 2\,\text{microgl. DNA copies reaction} / 2} * 22\ (\text{dilution factor to ml})$$

3.4. Statistical analyses

All viral RNA load statistics were performed using $\log_{10}$ transformed viral load values. Quantitative variables were described as the mean and standard deviation, and/or median. Correlations between two quantitative variables were measured by the Spearman correlation test. Descriptive statistics and linear regression lines were performed using Graph Pad Prism software (version 5.00.288). Agreement between the viral load results reported as $\log_{10}$ RNA copies/ml of UTM™ and $\log_{10}$ RNA copies/median number of cells was assessed using the Bland and Altman analysis.

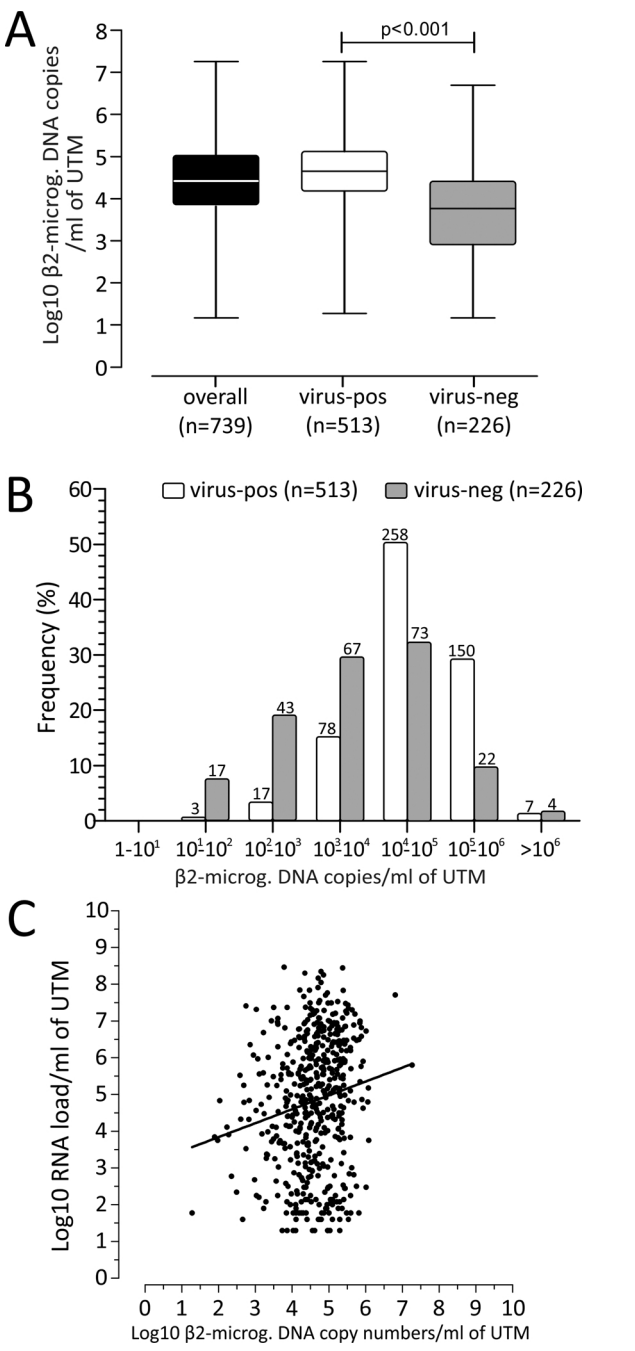

Fig. 1. Comparison of the number of cells measured in mid-turbinate flocked nasal swabs in the overall, virus-positive and virus-negative samples (A). Frequency distribution of the number of cells measured in respiratory virus-positive (white bars) *versus* virus-negative (grey bars) samples (B). Log-Log linear regression plots comparing the viral load and number of cells expressed as $\log_{10}$ β2-microgolubin DNA copy numbers/ml of UTM™ (C).

4. Results

A total of 739 samples were analyzed in this study. In 513 (69.4%) of these, at least one respiratory virus was detected, while 226 (30.6%) were negative. A total of 439/513 (85.6%) virus-positive samples were single samples collected from 439 patients, while 74/513 (14.4%) were follow-up samples collected from 29 patients. Among virus-positive samples, 190/513 (37.0%) were positive for HRV (median 4.85 $\log_{10}$ RNA copies/ml of UTM™, range 1.30 to 8.30 $\log_{10}$), 120/513 (23.4%) for influenza A (5.20 $\log_{10}$ RNA copies/ml of UTM™, range 1.30 to 8.47

log_{10}), 117/513 (22.8%) for RSV (5.54 log_{10} RNA copies/ml of UTM™, range 1.30 to 7.48 log_{10}) and 86/513 (17.8%) for hCoVs (3.82 log_{10} RNA copies/ml of UTM™, range 1.30 to 7.85 log_{10}).

Overall, a median of 4.42 log_{10} β2-microgolubin DNA copy number/ml of UTM™ was detected, range 1.17–7.26 log_{10} (Fig. 1A). A median higher number of cells was observed in virus-positive as compared to virus-negative samples (4.65 *vs* 3.76 log_{10} β2-microgolubin DNA copy number/ml of UTM™; $p < 0.001$). Whereas, no significant difference in the median cell number was observed when analyzing the samples according to the respiratory virus detected (HRV 4.65 log_{10} β2-microgolubin DNA copy number/ml of UTM™; influenza A 4.53 log_{10}; RSV, 4.85 log_{10} and hCoVs 4.55 log_{10}; $p > 0.05$) (Fig. S1).

Overall, in 486/513 (94.7%) virus-positive and 162/226 (71.7%) virus-negative samples, the number of cells measured ranged from 3.0 to 6.0 log_{10} β2-microgolubin DNA copy number/ml of UTM™ ($p < 0.001$; Fig. 1B). In addition, in 50.3% (258/513) of virus-positive samples, the number of cells measured was between log_{10} 4.0 and 5.0 as compared to 28.3% (73/226) in virus-negative samples ($p < 0.001$; Fig. 1B). As shown in Fig. 1C, the level of respiratory virus load (RNA copies/ml of UTM™) was independent of the amount of β2-microgolubin DNA (copy number/ml of UTM™; $p > 0.05$). For instance, in samples (n = 17) with high viral load ($> 10^6$ RNA copies/ml of UTM™) a low amount of human DNA ($< 10^4$ copy number/ml of UTM™) was detected.

In order to evaluate the use of cell number as a denominator for the standardization of viral load in respiratory samples, we compared viral load expressed as log_{10} RNA copies/ml of UTM™ and as log_{10} RNA copies/median number of cells (log_{10} 4.65 = 45,282 cells) in 513 virus-positive samples. As shown in Fig. 2A, a highly significant correlation was observed between the two methods of measuring viral load ($r = 0.89$, $p < 0.001$). Furthermore, this finding was also supported by the strength of agreement between the two methods, as illustrated by the linear regression analysis ($R^2 = 0.82$; Fig. 2A).

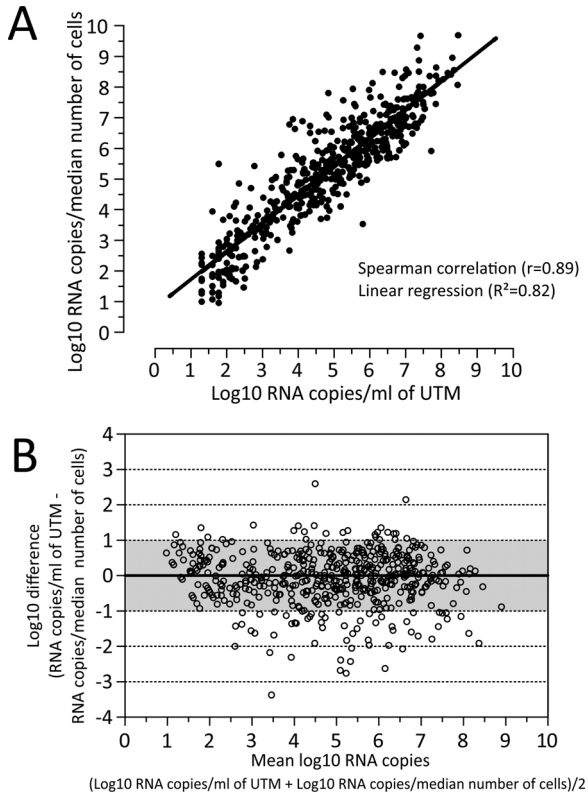

Fig. 2. Correlation analysis between viral loads expressed in log_{10} copies/ml of UTM™ or normalized to log_{10} copies/ median number of cells (A). Bland-Altman plots of log_{10} differences in viral RNA loads against the two methods of expressing results (B). The acceptability range (1 to -1 Log_{10} difference) is shaded in grey.

Agreement between viral load measurements was also evaluated by performing a Bland-Altman analysis with viral load expressed as log_{10} RNA copies/ml of UTM™ as a reference group (Fig. 2B). Overall, the mean log_{10} difference between the two methods of measuring viral load was -0.07 ± 0.77 (range −3.37 to +2.60). In detail, in 429/513 (83.6%) samples, the viral load difference fell within a ± 1 log_{10}, while in 258/513 (50.3%) the difference fell within a ± 0.5 log_{10}. In the remaining 84/513 (16.4%) samples, the viral load difference was < -1 log_{10} in 59/84 (70.2%), and $> +1$ log_{10} in 25/84 (29.8%) samples.

To assess the reproducibility of viral load expressed as log_{10} RNA copies/median number of cells, the viral load kinetics were compared between the two methods of expressing viral load in eight patients with a total of thirty-two follow-up samples (range 3–5 samples). No statistical difference was observed between the two methods ($p > 0.05$) and the mean difference was +0.22 ± 0.58 log_{10} (median +0.20, range -0.74 to 1.63). Indeed, comparable viral load kinetics were observed for all of the analyzed cases in which different respiratory viruses were detected (Fig. 3).

Finally, from twenty-one patients, two follow-up samples (n = 42) collected at a median time of 7 days (range 1–30 days) were available and thus the drop in viral load was compared between the two methods of expressing viral load. No statistical difference between the drop in viral loads expressed as log_{10} RNA copies/ml of UTM™ (median 1.48 range −0.90 to +5.22) and as log_{10} RNA copies/median number of cells was observed (median 1.00 range −2.40 to 4.23; $p > 0.05$).

5. Discussion

The accurate diagnosis of viral respiratory infections depends on the specimen collection as well as the diagnostic method used. Along these lines, single or multiplexed molecular assays are recognized as the gold standard for viral respiratory diagnosis [11]. Nevertheless, the collection and storage of respiratory samples remain critical steps in the diagnostic workflow. The flocked swab together with its specific transport medium is increasingly recognized as a valid alternative to commonly used specimen collection methods such as nasopharyngeal aspiration [12,13]. The main objectives of the present study were to measure the cellularity of mid-turbinate flocked nasal swab samples with well-characterized viral respiratory infections and to assess the validity of viral load normalization based on the median number of cells measured.

The median number of cells measured in mid-turbinate flocked nasal swab samples was similar to that observed in other recent studies [14,15]. In these studies, molecular cell quantification using real-time PCR was performed and therefore the data obtained could be compared with those presented here. In addition, our results were consistent with the findings observed in these two studies, in which samples positive for single or multiple viruses had a greater number of cells as compared to virus-negative nasal swabs [14,15]. In these studies, several reasons such as increased epithelial desquamation or a cytopathic effect induced *in vivo* have previously been hypothesized to explain this observation [14,15]. Additionally, these phenomena could be explained by local nasal immune system activation and recruitment of cell-mediated immunity during the early phase of infections when respiratory samples are usually collected. This hypothesis is also supported by the McNamara et al. study reporting a significant difference in the cellular response (mainly leukocytes) in bronchoalveolar lavage of patients with severe hRSV bronchiolitis as compared to controls without respiratory infection [16]. Similar results were also reported in another study where the cellularity of samples in RSV-positive samples was higher than that of controls [17].

Analyzing the cell number yield according to the different respiratory virus detected, no significant differences were observed (Fig. S1). This finding was in contrast with data presented by Bonnin et al.

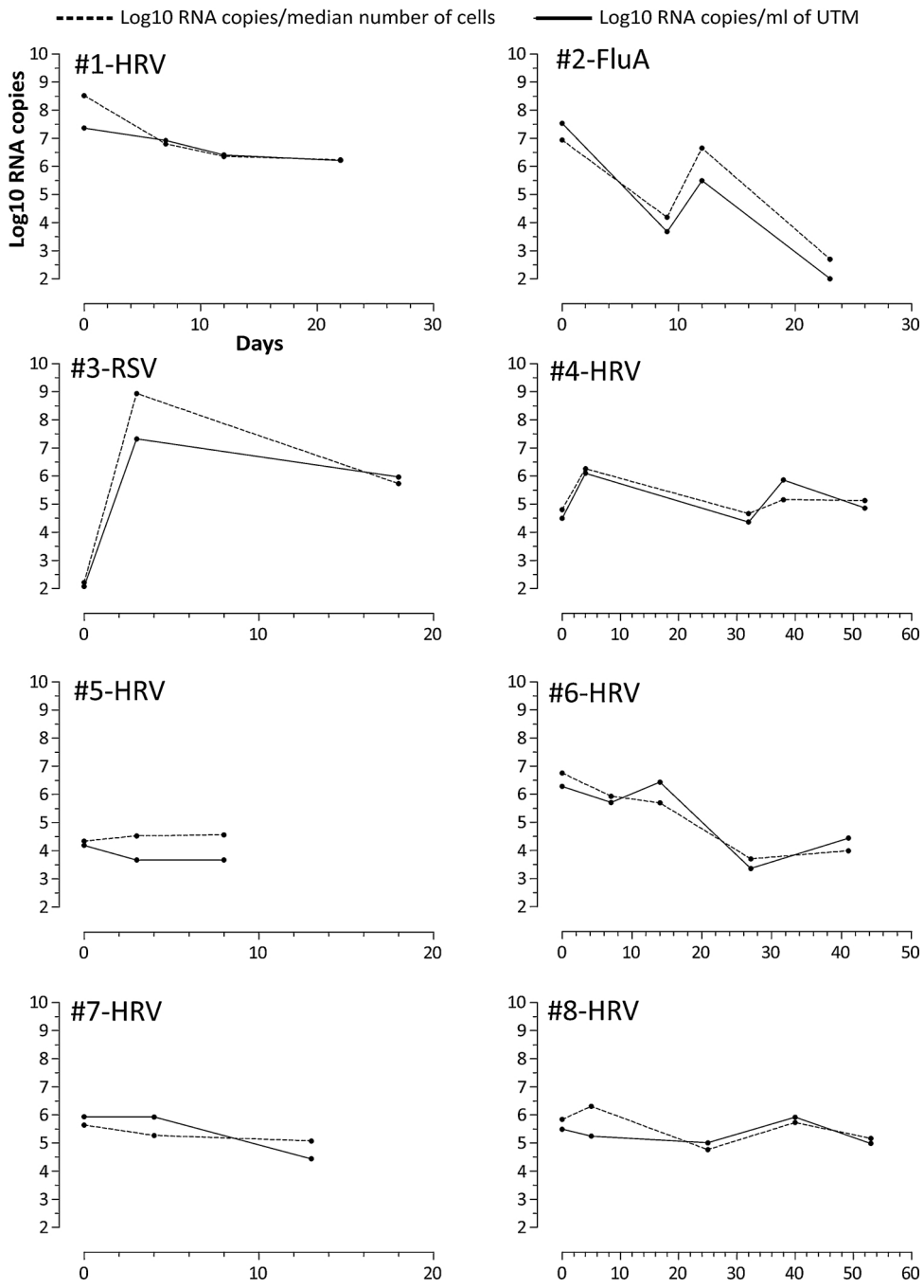

Fig. 3. Viral load kinetics measured in 8 episodes of respiratory infection expressed as log_{10} RNA copies/ml of UTM™ (solid line) and as log_{10} RNA copies/median number of cells (dashed line). HRV, human rhinovirus; FluA, influenza A virus; RSV, respiratory syncytial virus.

[10], where a statistically significant difference between RSV (n = 40) and rhinovirus/enterovirus (n = 106) and hMPV (n = 16) positive samples was observed. However, in the Bonnin report, the number of RSV and rhinovirus/enterovirus positive samples was lower than that analyzed in our study; thus, further studies with a larger number of virus-positive samples are needed to better understand the impact of cellularity on the detection of different respiratory viruses. In addition, the β2-microgolubin DNA content in the mid-turbinate flocked nasal swabs is independent of level of respiratory virus load as previously observed by others [18,19].

Use of the β2-microglobulin housekeeping gene allowed us to assess the possible effect of sample quality variation on the results. In detail, cell number was used as a denominator for the standardization of viral load in respiratory samples and evaluated by comparing the viral load expressed as log_{10} RNA copies/ml of UTM™ with log_{10} RNA copies/median number of cells. After adjusting the results for the cellular content using β2-microglobulin, similar results to the target copy number were obtained. In fact, in 83.6% of samples, the viral load difference fell within ± 1 $log_{10,}$ which is considered as an acceptable range of variability. In addition, a significant correlation was observed between the two methods of expressing viral load (R^2 = 0.82). Good correlation was also confirmed by analyzing the viral load kinetics in a follow-up series of samples collected from eight patients. No significant difference in the kinetics of viral shedding was observed using the different methods of expressing viral load. This finding was in keeping with a report by Loeb et al., which confirmed the utility of viral load normalization in respiratory samples using a housekeeping gene [20].

Our results, may also have implications for the standardization of

respiratory specimen collection such as nasopharyngeal aspirates or bronchoalveolar lavage that have intrinsic variability due to their dilution with saline, as well as differences in cellularity. It is important to mention that this study has several limitations. In addition to its retrospective nature, the results of this study could have been influenced by several factors that were not analyzed such as the sampling delay from the onset of symptoms and the sample integrity due to freeze-thaw events. However, the median number of cells measured in our study was similar to those observed in other studies [14,15] and therefore we have considered that the integrity of samples was maintained.

In conclusion, the results of the present study confirmed how mid-turbinate flocked nasal swab samples provide adequate cell numbers for the diagnosis of respiratory viruses. Larger cell numbers were recovered in virus-positive as compared to virus-negative samples. A good correlation between viral load normalized by volume (RNA copies/ml of UTM™) or by cell number (RNA copies/median number of cells) was demonstrated. This finding suggest that normalization seems to be unnecessary in clinical samples collected with mid-turbinate flocked nasal swabs and it would be only an introduction of additional costs and increase throughput.

Author contributions

AP: supervision, formal analysis, figure editing, writing original draft and manuscript revision; FG: data curation, investigation; FR: data curation, investigation; GC: data curation, investigation; FB: conceptualization and study design, manuscript revision.

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Competing interest

There were no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2018.07.010.

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