Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Cellular DNA quantification in respiratory samples for the normalization of viral load: a real need?

Antonio Pirallia,1, Federica Giardinia,1, Francesca Rovidaa, Giulia Campaninia, Fausto Baldantia,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

ARTICLE INFO

Keywords:
Respiratory viruses
Quantiﬁcation
Cell number
Flocked mid-turbinate nasal swabs
Viral load

ABSTRACT

Background: Respiratory tract infections have an enormous social and economic impact, with a 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-microglobulin 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.

https://doi.org/10.1016/j.jcv.2018.07.010
Received 10 May 2018; Received in revised form 25 July 2018; Accepted 26 July 2018
1386-6532/ © 2018 Published by Elsevier B.V.
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 log10 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-microglobulin 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 log10 copies) per median number of cells recovered in positive samples (4.65 log10 β2-microglobulin DNA copy number/ml of UTM™), and calculated as follows:

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

3.4. Statistical analyses

All viral RNA load statistics were performed using log10 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 log10 RNA copies/ml of UTM™ and log10 RNA copies/median number of cells was assessed using the Bland and Altman analysis.

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 log10 RNA copies/ml of UTM™, range 1.30 to 8.30 log10), 120/513 (23.4%) for influenza A (5.20 log10 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$^6$ 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™. In 486/513 samples, the number of cells measured was between log$_{10}$ 4.0 and 5.0 as compared to virus-negative samples (4.65 log$_{10}$ and hCoVs 4.55 log$_{10}$; p > 0.05) (Fig. S1). This finding was in contrast with data presented by Bonnin et al. [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].

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.
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-microglobulin 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.

Funding

The analysis and interpretation of data for this project were funded by the Ministero della Salute, Fondazione IRCSS Policlinico San Matteo, Ricerca Corrente (grant no. 80622). The study design, experimental analysis, and manuscript editing were also supported by Copan Italia SpA Brescia, Italy.

Competing interest

There were no conflict of interest.

Acknowledgements

We thank Daniela Sartori for careful preparation of the manuscript and Lauren Kelly for English revision.

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.

References

[1] H.V. Fineberg, Pandemic preparedness and response–lessons from the H1N1 influenza of 2009, N. Engl. J. Med. 370 (2014) 1335–1342, https://doi.org/10.1056/NEJMsa1208402.

[2] T. Heikinnen, M. Shenoy, R.M. Goldblum, T. Chonnaintree, Free secretory component as a standardization protein for nasopharyngeal specimens from children with upper respiratory tract infection, Acta Paediatr. 88 (1999) 150–153, https://doi.org/10.1111/j.1651-2227.1999.th01073.x.

[3] T. Heikinnen, J. Marttila, A.A. Salmi, O. Ruskanen, Nasal swab versus nasopharyngeal aspirate for isolation of respiratory viruses, J. Clin. Microbiol. 40 (2002) 4337–4339, https://doi.org/10.1128/JCM.40.11.4337-4339.

[4] P. Daley, S. Castriciano, M. Cherney, S. Mejia, Comparison of flocked and rayon swabs for collection of respiratory epithelial cells from uninfected volunteers and symptomatic patients, J. Clin. Microbiol. 44 (2006) 2265–2267, https://doi.org/10.1128/JCM.02655-05.

[5] S. Esposito, C.G. Molteni, C. Daleno, A. Valzano, C. Tagliaferi, C. Galeone, et al., Collection by trained pediatricians or parents of mid-turbinate nasal flocked swabs for the detection of influenza viruses in childhood, Virol. J. 7 (85) (2010), https://doi.org/10.1186/1743-422X-7-85.

[6] O.E. Larios, B.L. Coleman, S.J. Drews, T. Mazzulli, B. Borgundvaag, K. Green, et al., Self-collected mid-turbinate swabs for the detection of respiratory viruses in adults with acute respiratory illnesses, PLoS One 6 (2011), https://doi.org/10.1371/journal.pone.01335–e21335.

[7] A. Piralla, F. Baldanti, G. Gerna, Phylogenetic patterns of human respiratory picornavirus species, including the newly identified group C rhinoviruses, during a 1-year surveillance of a hospitalized patient population in Italy, J. Clin. Microbiol. 49 (2011) 373–376, https://doi.org/10.1128/JCM.01814-10.

[8] A. Piralla, G. Lunghi, E. Percivalle, C. Viganò, T. Nasta, L. Pugni, et al., FilmArray® respiratory panel performance in respiratory samples from neonatal care units, Diagn. Microbiol. Infect. Dis. 79 (2014) 183–186, https://doi.org/10.1016/j.diagmicrobio.2014.02.010.

[9] F. Watzinger, M. Suda, S. Preuner, R. Baumgartinger, K. Ebner, L. Baskova, et al., Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients, J. Clin. Microbiol. 42 (2004) 5189–5196, https://doi.org/10.1128/JCM.42.11.5189-5196.2004.

[10] M. Smieja, S. Castriciano, S. Garruthers, G. So, S. Chong, K. Luintela, et al., Development and evaluation of a flocked nasal midturbinate swab for self-collection in respiratory virus infection diagnostic testing, J. Clin. Microbiol. 48 (2010) 3340–3342, https://doi.org/10.1128/JCM.02235-09.

[11] K.E. Templeton, S.A. Scheltinga, W.C. van den Eeden, A.W. Graffelman, P.J. van den Broek, E.C. Claas, Improved diagnosis of the etiology of community-acquired pneumonia with real-time polymerase chain reaction, Clin. Infect. Dis. 41 (2005) 345–351, https://doi.org/10.1086/381588.

[12] L. Li, Q.Y. Chen, Y.Y. Li, Y.F. Wang, Z.F. Yang, N.S. Zhong, Comparison among nasopharyngeal swab, nasal wash, and oropharyngeal swab for respiratory virus detection in adults with acute pharyngitis, BMC Infect. Dis. 13 (2013) 281, https://doi.org/10.1186/1471-2334-13-281.

[13] P.K. Munywoki, F. Hamid, M. Mutenga, S. Welch, P. Cane, D.J. Nokes, Improved detection of respiratory viruses in pediatric outpatients with acute respiratory illness by real-time PCR using nasopharyngeal flocked swabs, J. Clin. Microbiol. 49 (2011) 3365–3367, https://doi.org/10.1128/JCM.02231-10.

[14] P. Bonnin, F. Miszczak, N. Kin, C. Resa, J. Dina, S. Gouarin, et al., Study and interest of cellular load in respiratory samples for the optimization of molecular virological diagnosis in clinical practice, BMC Infect. Dis. 16 (2016) 384, https://doi.org/10.1186/s12879-016-1730-9.

[15] A.N. Asalbeh, D.M. Whiley, S. Bialasiewicz, S.B. Lambert, R.S. Ware, M.D. Nissen, et al., Nasal swab samples and real-time polymerase chain reaction assays in community-based, longitudinal studies of respiratory viruses: the importance of sample integrity and quality control, BMC Infect. Dis. 14 (2014) 15, https://doi.org/10.1186/1471-2334-14-15.

[16] P.S. McNamara, P. Rion, A. Selby, C.A. Hart, R.L. Smyth, Bronchoalveolar lavage cellularity in infants with severe respiratory syncytial virus bronchiolitis, Arch. Dis. Child. 88 (2003) 922–926, https://doi.org/10.1136/adc.88.10.922.

[17] J. Heidema, M.V. Lukens, W.W. van Maren, M.E. van Dijk, H.G. Otten, A.J. van Vught, et al., CD8 + T cell responses in bronchoalveolar lavage fluid and peripheral blood mononuclear cells of infants with severe primary respiratory syncytial virus infections, J. Immunol. 179 (2007) 8410–8417, https://doi.org/10.4049/jimmunol.179.12.8410.

[18] L. Van Wesenbeeck, H. Meeuws, D. D’Haese, G. Iapks, L. Houpis, M. Van Ranst, et al., Sampling variability between two mid-turbinate swabs of the same patient has implications for influenza virus load monitoring, Virol. J. 11 (2014) 233, https://doi.org/10.1186/1743-422X-11-233.

[19] M.K. Akmatov, A. Gatzenemeier, K. Schoght, F. Pesler, Equivalence of self- and staff-collected nasal swabs for the detection of viral respiratory pathogens, PLoS One 7 (2012) e48508, https://doi.org/10.1371/journal.pone.0048508.

[20] M. Loeb, P.K. Singh, J. Fox, M.L. Russell, K. Fabburaraj, D. Zarrat, et al., Longitudinal study of influenza molecular viral shedding in Hutterite communities, J. Infect. Dis. 206 (2012) 1078–1084, https://doi.org/10.1093/infdis/jis450.