The effect of assay type and sample matrix on detected cytokine concentrations in human blood serum and nasal lavage fluid

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ABSTRACT

Cytokine concentrations in biological fluids are widely used markers for activation of immunological processes. Confirming the reproducibility of measurements is important, especially in longitudinal or multicenter studies where time between analyses or different analyzing laboratories increases the intra-assay variability. In this study, the reproducibility of the cytokine analysis conducted with different assay platforms was studied by comparing the results of two cytokines [interleukin (IL)-6 in serum and nasal lavage fluid (NAL) and IL-8 in NAL] analyzed with Meso Scale Discovery (MSD) ultra-sensitive single and multiplex assay kits ($n = 76$). In addition, the difference in cytokine levels between two biological sample matrices was studied by comparing the results of altogether 9 cytokines [IL-6, IL-2, IL-8, IL12p70, IL-1β, granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon (IFN)γ, IL-10 and tumor necrosis factor (TNF)α] measured from serum and NAL of the same study subjects ($n = 460$).

The results show that the cytokine concentrations analyzed with single and multiplex assays are concordant but not equal. Comparison of the different matrices revealed that cytokine concentrations in serum do not correspond with concentrations detected in nasal lavage fluid.

It can be concluded that comparability of the results from single and multiplex analysis of cytokines is high, but the concentrations should not be compared directly with each other. The differences between concentrations analyzed from serum and nasal lavage fluid indicate that the levels are specific for each matrix and represent distinct immunological conditions.

ABBREVIATIONS

ELISA, enzyme linked immunosorbent assay;
IA, immunoassay;
RT-PCR, reverse transcription polymerase chain reaction;
MSD, Meso Scale Discoveries;
PBS, Phosphate-Buffered Saline;
IL, interleukin;
IFN, interferon;
GM-CSF, granulocyte–macrophage colony-stimulating factor;
TNF, tumor necrosis factor;
NAL, nasal lavage

1. INTRODUCTION

Cytokines are recognized as important molecules in cellular signaling in both healthy and diseased individuals [1], [2] and [3]. However, the complexity and diverse nature of these proteins calls for techniques able to analyze simultaneously multiple cytokines or composition of cytokine networks rather than concentrations of single analytes. During the last decade, multiplex technology has enabled the analysis of large panels from even smaller sample volumes, widening significantly the scope of cytokine research. Before this technology is fit for use in study settings involving multiple centers or longitudinal design, the reproducibility of the method has to be proven. There are several reports on comparison of multiplex methods against the enzyme-linked immunosorbent assay (ELISA) showing good agreement in culture supernatants [4] and [5] but deteriorating quantitative agreement in more complex sample matrices [6] and [7]. Typically, the absolute levels of cytokines can be affected, e.g., by choice of supplier for the reagents [8], which complicates the comparison between different platforms even further.

Techniques for measuring cytokines from biological liquids include several methods utilizing antibody–antigen reactions, e.g., ELISA, other immunoassays (IA), multiplex bead assay and multiplex suspension array, and also methods detecting the gene expression level, e.g., RT-PCR [9]. ELISA and IA require a large amount of sample whereas multiplex bead assay or multiplex suspension array detects numerous proteins simultaneously from low sample volume [9], [10] and [11]. The MSD kit assays used in this study are based on sandwich immunoassay technique, where cytokines in liquid sample bind to capture antibodies immobilized on the working electrode surface and the labeled detection antibodies in turn bind the captured cytokine. MSD SULFO-TAG™ label is electrochemiluminescent

compound and it emits light when voltage is applied to the plate electrode, providing high sensitivity for the detection of low level of cytokines [12].
There are relatively few data available on variation of cytokine concentrations in different biological matrices, but studies with healthy volunteers show that concentrations vary according to sample material [9], [11] and [13]. Cytokines are typically produced locally, and the capability to produce certain cytokines is cell-specific, thus the concentrations of cytokines are likely to differ according to the sampling material and site. Cytokines have been measured, e.g., from blood, serum, plasma, urine, saliva, conjunctival sac fluid, gingival crevicular fluid, cultured and collected blood cells, nasal lavage fluid, induced sputum, bronchoalveolar fluid and exhaled breath condensate [9], [14], [15] and [16]. In addition to the differences in the production of cytokines at different sites, also the sample matrix itself may affect the concentrations by hindering the analysis. This is evident especially when analyzing complex matrices such as serum or highly diluted samples such as exhaled breath condensate or nasal lavage fluid [14] and [17]. The improved sensitivity of the assays has made it possible to detect significant differences in cytokine profiles also at subclinical level, although the biological relevance of differences in very low concentrations is questionable.
The aim of this study was to determine the reproducibility of the cytokine analysis in multiplex and singleplex assays from the same manufacturer and to compare the cytokine levels in corresponding serum and nasal lavage fluid samples. The collected data provides crucial information for assessing the reliability of the data obtained with multiplex platform and usability of the methodology, e.g., in multicenter research projects.

2. MATERIALS AND METHODS

2.1. Sample collection

The analyzed serum and nasal lavage samples were collected for the multinational research project “Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches (HITEA)”. As a part of the project, a cross-sectional and longitudinal study of teachers’ health in moisture damaged and reference school buildings in three European countries (Spain, The Netherlands and Finland) was conducted. Samples of blood and nasal lavage fluid were collected in three sampling campaigns within a year; April–June 2009 (before the summer holidays), August–September 2009 (after summer holidays) and January–March 2010.
The blood and nasal lavage samples were taken from the same teachers by a centrally trained healthcare professional during the school visits. All samples were transported within 8 h at +4 °C to the processing laboratory. For each study subject, one 8.5 ml serum tube with separating gel was filled and allowed to stand for 30 min. The serum was separated by centrifugation (1780 × *g*, 15 min, room temperature), after which it was aliquotted and frozen (−70 °C). The nasal lavage samples were taken by lavaging the nasal cavities with altogether 9 ml of warm physiological solution

[Phosphate-Buffered Saline (PBS), Gibco®, Life Technologies, Paisley, UK] [18]. The samples were centrifuged (425 × *g*, 10 min, room temperature) and the separated supernatant was aliquotted and frozen (−70 °C).

2.2. Cytokine assay

The cytokine analyses were completed in Inhalation Toxicology Laboratory at University of Eastern Finland using Meso Scale Discovery (MSD) Sector Imager™ 2400A with Discovery Workbench® 3.0 software. The samples were analyzed both with MSD® Human IL-6 Ultra-Sensitive Kit and MSD® Human TH1/TH2 10-Plex Ultra-Sensitive Kit (for IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13 and TNFα), both purchased from MSD (Rockville, MD, USA). First, a subset (n = 76) of serum and nasal lavage samples were analyzed according to manufacturer's instructions and using reagents provided with the kit. Assay diluent was added to all wells of a pre-coated 96-well plate and samples, standards and controls were incubated for 2 h in room temperature on an orbital shaker. After washing three times with wash buffer (PBS + 0.05% Tween 20), each analyte bound to a specific spot was further conjugated by adding detection antibody and incubating for 1 h in room temperature. After washing, the read buffer was added to each well and the amount of analyte was assessed by detecting the electrochemiluminescence signal with a sensitive camera. The concentration of the analyte was calculated by comparing the results to the standard curve reconstituted in the provided assay diluent.
Based on the results of the subset, nine cytokines (including one not tested in the subset, GM-CSF) were selected to be measured from the whole set of serum (n = 470) and nasal lavage (n = 489) samples and the method was modified to improve sensitivity and repeatability of the analysis. Briefly, a blocking step was added to minimize the nonspecific binding of antibodies, and the incubation time with samples was increased to overnight (+4 °C, shaker) to improve the sensitivity of the assay. In serum samples, 9 cytokines (IL-2, IL-8, IL12p70, IL-1β, GM-CSF, IFNγ, IL-6 IL-10 and TNFα) were analyzed using MSD® Human ProInflammatory 9-Plex Ultra-Sensitive Kit, whereas in nasal lavage samples, 8 cytokines (IL-2, IL12p70, IL-1β, GM-CSF, IFNγ, IL-6, IL-10, TNFα) were analyzed with MSD® Human ProInflammatory Custom 8-Plex Ultra-Sensitive Kit and IL-8 was measured using MSD® Human IL-8 Ultra-Sensitive Kit. A separate analysis of IL-8 from the nasal lavage samples was done because in some cases the high concentrations of IL-8 appeared to disturb the analysis of other cytokines in the multiplex-system. The detection limit was defined for each cytokine and for each plate separately by using the Meso Scale Discovery Sector Imager™ 2400A with Discovery Workbench® 3.0 software. The lower limit of detection was the calculated concentration of the signal that is 2.5 standard deviations over the zero calibrator of the kit (Table 1). The samples were measured in duplicates, thus the resulting concentration is the mean of the two replicate samples.

[TABLE 1]

2.3. Statistical analysis

The data description values and statistical tests were computed by IBM SPSS Statistics Version 19. The data was not normally distributed, thus non-parametric Wilcoxon matched-pairs signed-ranks test and Spearman rank correlation were used to analyze the differences and strength of association between the groups. The values below detection range were included in the statistical analysis, but the sensitivity analysis was done also by analysing the data with only values above the detection limit.

3. RESULTS

3.1. Effect of analysis method

The effect of the analysis method was studied in a subset of 76 samples by comparing the concentrations of IL-6 analyzed with single- and multiplex assays from serum and nasal lavage samples as well as IL-8 analyzed from nasal lavage samples. Strong linear rank correlation was found between concentrations analyzed by single and multiplex method in nasal lavage fluid (IL-6: $r = 0.95$, $p < 0.001$, $n = 70$ and IL-8: $r = 0.77$, $p < 0.001$, $n = 76$). Almost as high rank correlation was detected in IL-6 analyzed by the different methods from serum ($r = 0.57$, $p < 0.001$, $n = 70$) (Fig. 1). However, in the nasal lavage fluid the concentrations measured by multiplex assay were slightly lower than concentrations measured with singleplex assay, increase of median concentrations being from 0.96 to 1.12 pg/ml (17%, $p = 0.014$) for IL-6 and from 110 to 149 pg/ml (35%, $p < 0.001$) for IL-8. On the other hand, in serum samples the IL-6 concentrations measured with multiplex assay were typically higher compared to singleplex assay (0.64 vs. 0.38 pg/ml, 68%, $p < 0.001$) (Fig. 2). The result remained the same also when the values below the detection limit were excluded from the statistical analyses.

3.2. Effect of sample matrix

The effect of sample matrix was studied in the complete dataset consisting of concentrations of nine cytokines in 460 corresponding pairs of serum and nasal lavage fluid samples. The levels of the cytokines were generally low, medians ranging from 0.46 to 8.51 pg/ml in serum and 0.04 to 5.50 pg/ml in nasal lavage fluid, the only exception being IL-8 with median concentration of 179 pg/ml. In serum samples more than 80% of the values of all 9 analyzed cytokines were in the detection range, whereas in nasal lavage fluid only three cytokines (IL-1β, IL-6 and IL-8) had more than 80% of samples in detection range. However the median levels and range of these three cytokines were higher in nasal lavage than in serum (Table 2).

The concentrations of the cytokines measured from serum and nasal lavage fluid did not correlate with each other, with the exceptions of IL-6, IFNγ and TNFα, which showed weak but statistically significant correlation. Most of the measured cytokines had a low amount of samples in detection range, which affects the reliability of the results. After excluding the values under the detection limit from the analysis, only

the correlations for IL-6 and IFNγ reached statistical significance (Table 3). The cytokine concentrations were detected to be significantly different in serum samples in comparison to the nasal lavage samples. The biological sample type where the concentration was higher varied depending on the individual cytokines: concentrations were higher in serum compared to nasal lavage fluid when GM-CSF, INF-γ, IL-10, IL-12p70, IL-2 and TNF-α were tested ($p < 0.001$), whereas the concentrations of IL-1β, IL-6 and IL-8 were detected to be higher in nasal lavage than in serum ($p < 0.001$).

[TABLE 2]

[FIGURE 1]

4. DISCUSSION

It is well known that in addition to selected sample material, the cytokine profile and cytokine concentrations can be affected by personal differences such as health status, gender, age and ethnicity, all adding to the variability in the results of cytokine analysis [9], [11], [13], [14] and [19]. Also the differences between commercial assays of separate manufacturers and analysis methods can make the results from different studies incomparable [20], [21] and [22]. Other factors possibly causing variability to the results include lot-to-lot variation, different analysing laboratories, different storage conditions and handling of biological samples [19], [20] and [23]. Our study shows that results of cytokine measurements obtained with single and multiplex platforms from the same manufacturer are comparable when relative changes are considered, but quantitative comparison of samples is not possible due to the differences in absolute values. This finding is supported also by a previous study, where a high correlation between cytokines analyzed by MSD® multiplex and singleplex kits was found but due to proportional bias, the means of 2 out of 4 analyzed cytokines differed significantly [22]. In serum samples the IL-6 values were found to be slightly higher when measured with multiplex platform, whereas in nasal lavage the situation was the other way around both for IL-6 and IL-8. Noting that in serum the IL-6 levels were close to the detection limit, we assume that the slight increase of signal in the multiplex plates originates from interference of the neighboring spots in the same well. Interestingly, when comparing the values measured with the different platforms, a group of NAL samples showed somewhat higher IL-8 values measured with multiplex assay, although the correlations between the two platforms within this group and in rest of the data were similar. We found no apparent reason for such grouping of the data, thus the possible cause of this (e.g., interference from other analytes) needs to be studied further.

[TABLE 3]

[FIGURE 2]

Furthermore, we detected that the cytokine concentrations were significantly different in serum compared to nasal lavage fluid, and the biological matrix where the concentration was higher varied depending on the cytokine under consideration. The difference in cytokine concentration between biological matrices indicates that they represent distinct locations or situations in immunological function of the body, e.g., cytokine concentration in serum represents systemic response whereas cytokine concentrations in nasal lavage fluid reflects the local situation in the upper airways.

In addition to variation between different sample types – i.e., here nasal lavage vs. blood serum – caused by local production of cytokines, the sample matrix may have endogenous or exogenous factors, soluble receptors, carriers, binding proteins and antagonists that may interact with cytokines or with antibodies of the analysing method and thus affect the result of the analysis [19].

For the repeatability of the results, it is important that multiplex analysis is able to perform repeated measures of the cytokine panels in the same subjects under the same conditions. In addition to the lack of validation, the multiplex analyses are sensitive to the changes in protein content of the sample and the multitude of antibodies in assays can react with each other and with the other components of samples. There may also be problems with intra-well interference when two analytes have very different concentrations or with variability of the capture antibody spot size and density when plate manufacturing process has quality issues [10], [23] and [24]. This was seen also in our analysis, where in some cases the high concentrations of IL-8 interfered with the simultaneous detection of other cytokines in nasal lavage fluid. These problems have to be solved before the reliability and reproducibility of the multiplex assays reach the level of validated ELISA assay.

In conclusion, due to the high correlation between cytokine concentrations measured with different platforms, the results obtained with single- and multiplex kits can be compared with each other, but by using rank values or relative changes rather than absolute values. The lack of correlation between analyte levels in serum and nasal lavage fluid further confirms that cytokine concentrations should be considered to be matrix-specific and indicators of distinct immunological conditions.

Acknowledgements

This work was supported by the European Commission as part of HITEA (Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches), Grant agreement no. 211488 under the Seventh Framework Programme, Topic ENV.2007.1.2.1.1. ‘Indoor air pollution in Europe: An emerging environmental health issue’.

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TABLES AND FIGURES

Table 1
The average detection limits with 95% confidence interval (CI) for nine cytokines from blood serum and nasal lavage fluid samples (NAL) analyzed using Meso Scale Discovery Sector Imager™ 2400A with Discovery Workbench® 3.0 software.

Cytokine	SERUM (pg/ml), $n = 12$		NAL (pg/ml), $n = 13$	
	Average	95% CI	Average	95% CI
GM-CSF	0.26	0.18–0.34	0.17	0.13–0.21
IFN-γ	0.77	0.49–1.05	0.58	0.46–0.70
IL-10	0.54	0.34–0.73	0.24	0.19–0.29
IL-12p70	0.41	0.27–0.56	0.43	0.33–0.53
IL-1β	0.16	0.14–0.19	0.17	0.10–0.25
IL-2	0.25	0.19–0.30	0.24	0.18–0.30
IL-6	0.11	0.10–0.13	0.09	0.06–0.12
IL-8	0.05	0.04–0.06	0.11	0.07–0.16
TNF-α	0.55	0.48–0.61	0.31	0.17–0.46

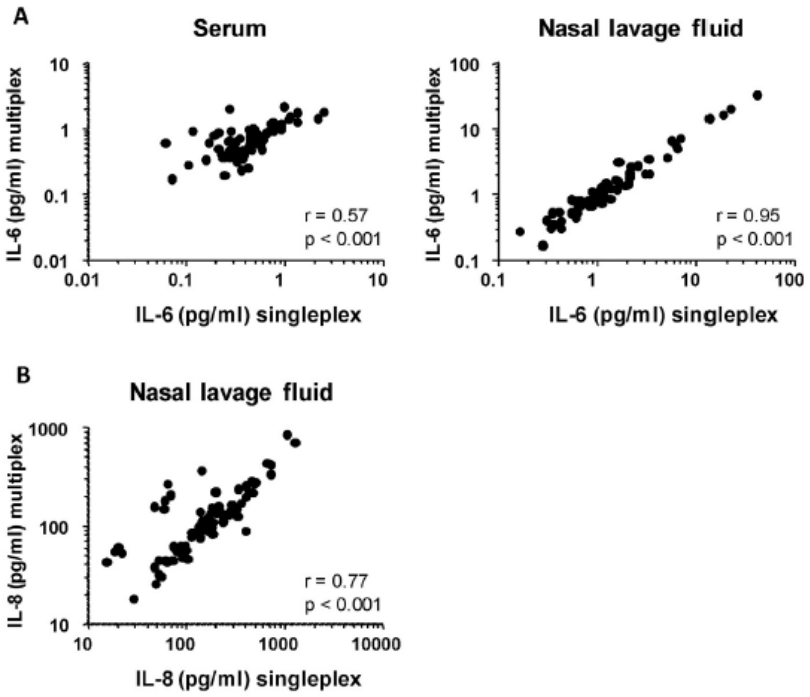

Fig. 1. Correlation between singleplex and multiplex methods. Scatterplot of concentrations (pg/ml) of (A) IL-6 in blood serum and nasal lavage fluid (NAL) and (B) IL-8 in NAL analyzed with MSD® single and multiplex assays. *r*, Spearman rank correlation coefficient; *p*, statistical significance of correlation.

Huttunen, K., Tiihonen, K., Roponen, M., Heederik, D., Zock, J.P., Täubel, M., Hyvärinen, A., Hirvonen, M.R. The effect of assay type and sample matrix on detected cytokine concentrations in human blood serum and nasal lavage fluid. Journal of Pharmaceutical and Biomedical Analysis: 2014, 96(aug.), 151-155

Table 2
Descriptive statistics of nine cytokines analyzed from (A) serum ($n = 470$) and (B) nasal lavage fluid (NAL, $n = 489$).

	% of samples in detection range	Min	Percentiles			Max
			25th	50th	75th	
(A) Serum						
GM-CSF	91	0.10	0.45	0.66	1.18	25.7
IFNγ	90	0.11	1.15	1.77	2.63	35.5
IL-10	100	1.26	3.44	5.68	11.08	455
IL-12p70	96	0.28	1.06	2.01	4.42	425
IL-1β	93	0.05	0.36	0.51	0.75	11.4
IL-2	81	0.02	0.31	0.46	0.68	14
IL-6	100	0.17	0.45	0.61	0.92	164
IL-8	100	1.48	4.67	6.70	9.88	107
TNFα	100	4.28	7.13	8.51	10.1	125
(B) NAL						
GM-CSF	12	0.00	0.03	0.10	0.13	1.72
IFNγ	< 0.1	0.00	0.00	0.10	0.17	111
IL-10	30	0.00	0.06	0.16	0.35	120
IL-12p70	< 0.1	0.00	0.00	0.04	0.12	0.75
IL-1β	99	0.00	2.42	5.50	12.6	213
IL-2	0.1	0.00	0.02	0.07	0.12	1.27
IL-6	99	0.03	0.61	1.19	2.62	383
IL-8	100	15.5	90.2	179	394	3380
TNFα	50	0.00	0.16	0.33	0.61	58.0

Table 3
Spearman rank correlation of cytokine concentrations in serum and nasal lavage fluid ($n = 460$). Statistically significant correlation ($p \leq 0.05$) is indicated with bold font.

Cytokine	Complete data		Data in detection range		n (%) of pairs in detection range
	r	p	r	p	
GM-CSF	0.08	0.071	−0.11	0.435	50(11)
IFNγ	0.13	0.005	0.48	0.026	21(4.6)
IL-10	0.03	0.484	0.15	0.083	140(30)
IL-12p70	0.09	0.069	–	–	2(0.4)
IL-1β	−0.04	0.418	−0.06	0.218	425(92)
IL-2	0.041	0.383	−0.15	0.460	26(5.7)
IL-6	0.16	0.001	0.16	0.001	455(99)
IL-8	0.07	0.116	0.07	0.116	460(100)
TNFα	0.14	0.003	0.05	0.482	223(48.5)

r, correlation coefficient; p, statistical significance (p-value); n, number.

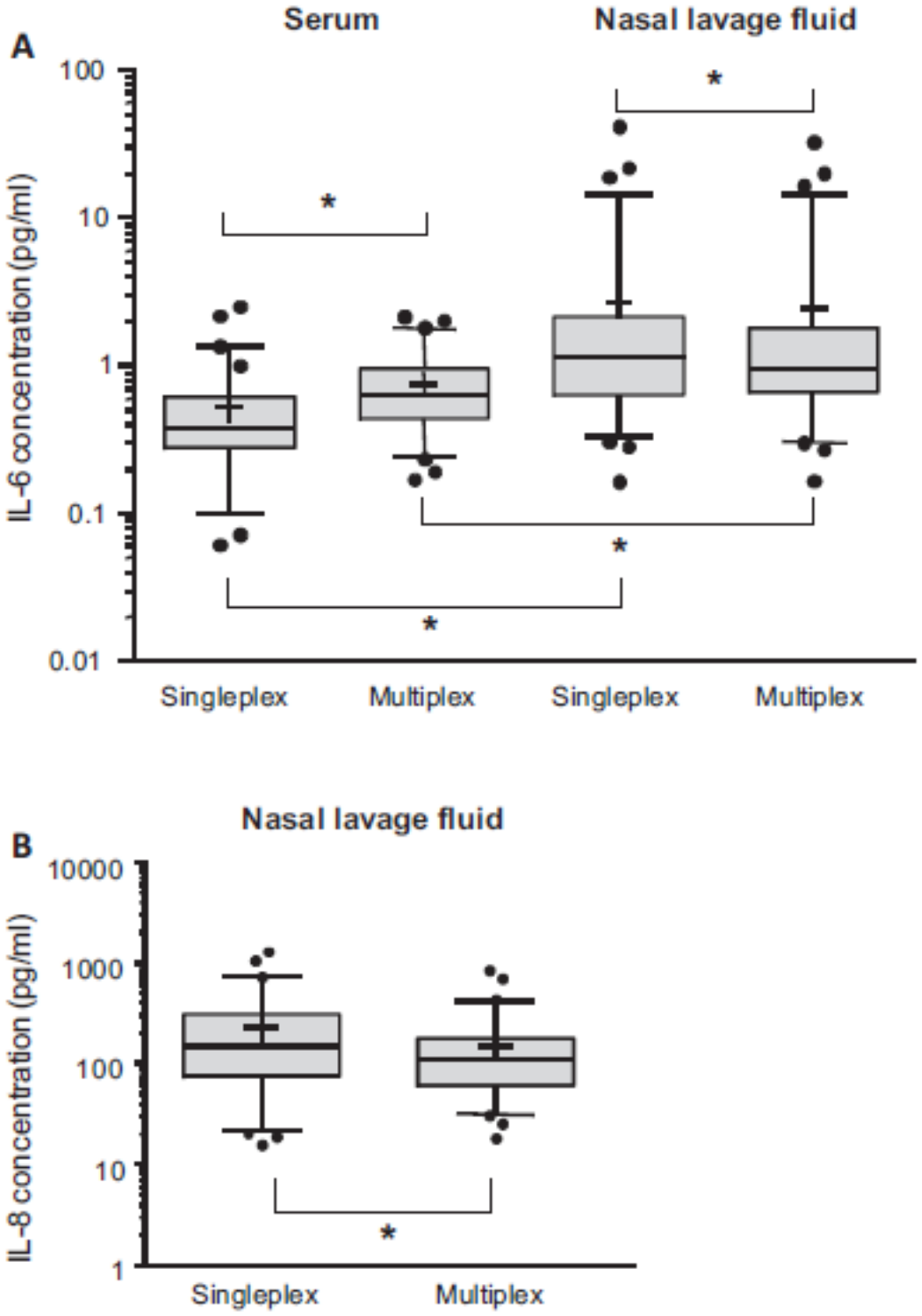

Fig. 2. The effect of analysis method on measured cytokine concentrations. The distribution of (A) IL-6 concentration (pg/ml) in serum and nasal lavage fluid and (B) IL-8 concentration (pg/ml) in nasal lavage fluid analyzed by single and multiplex assays. Whiskers, 5th and 95th percentiles; +, mean; •, data point outside of 5th and 95th percentiles; *, statistically significant difference between matched pairs in Wilcoxon matched-pairs signed-ranks test ($p < 0.05$).