HHS Public Access

Author manuscript

J Immunol Methods. Author manuscript; available in PMC 2018 December 03.

Published in final edited form as:

J Immunol Methods. 2017 August ; 447: 65–70. doi:10.1016/j.jim.2017.04.010.

A PCR-based method for quantifying neutrophils in human nasal secretions

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Abstract

Neutrophil recruitment to the nasopharynx (NP) is a central event in resolution of NP-initiated microbial infections. A vigorous neutrophil response in infected tissues is also associated with the outcome of adverse tissue pathology. Therefore, differences in infection-induced tissue neutrophil numbers may correlate with pathogenesis events. Existing methods of quantifiying neutrophils require evaluation of NP samples within hours of procurement as flow cytometry based cell quantification methods require live neutrophil cells. Therefore, we developed a novel RT-PCR method that could reliably quantify neutrophil counts in frozen NP wash samples. mRNA transcripts of the genes encoding CD16, CD18, and CD62L were identified as neutrophil-specific in NP samples and not significantly variable in response to stimulation by heat killed bacteria, and can be used to derive an accurate assessment of neutrophil content in a sample even in the presence of epithelial cells. Using flow cytometry as a comparator, the method was validated in human NP wash samples. We conclude that this PCR-based method should prove useful for providing a quantitative estimate of neutrophil recruitment to the NP during infection and pathogenesis.

1. Introduction

Neutrophils are the most abundant leukocyte in human blood. They are the first cells recruited to the site of an infection or injury (Kolaczkowska and Kubes, 2013), constituting the “front line” of immune defense. Neutrophils migrate up concentration gradients of chemokines such as IL-8, before being arrested by selectins on the vascular endothelial surface and extravasating into tissue (Kobayashi, 2008; Nicu and Loos, 2016). During an active infection in mucosal tissues, resident innate cells such as macrophages effectively promote recruitment of neutrophils that are needed to clear infection (Farrera and Fadeel, 2013; Murray and Wynn, 2011). Following infiltration, neutrophils are the most powerful innate effector cell type for pathogen phagocytosis and clearance. While neutrophils recruitment correlates with the prevention of infection, neutrophil recruitment in infected tissues is also associated with pathology and tissue damage. Therefore, a protective host

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response to an infection must orchestrate a balanced innate effector response that is more predominantly associated with infection clearance. Flow cytometry and histological staining remain the two most important methods for assessing neutrophil numbers during infection or inflammatory disease (Heron et al., 2012). However, histological staining can yield only a qualitative estimate of cell abundance, and flow cytometry must be performed on fresh samples because notoriously-fragile neutrophils rupture during various freezing procedures.

Quantitative PCR (qPCR) is a powerful and well-established technique for measuring absolute or relative differences in the quantities of DNA or RNA sequences in a sample based on the time to amplify a specific sequence past a given critical threshold (CT) of intensity with the use of fluorescent dyes (Hunter et al., 2010). A lower CT value corresponds to a greater quantity of target sequence in a sample. Since measurements are sensitive to the total DNA content of a sample in addition to the cells' activation status, constitutively expressed reference or "housekeeping" genes such as *GAPDH, 18S* rRNA, or *ACTB* (β-actin) are examined simultaneously with the sequences of interest to normalize results between samples (Jacob et al., 2013).

Here, we demonstrate that the neutrophil content of frozen human NP wash samples can be accurately determined by performing quantitative PCR on *FCGR3A* (CD16), *LTBR* (CD18), and *SELL* (CD62L) immune markers, using *18S* rRNA and *GAPDH* as reference genes. These genes are stably expressed even in the face of microbial stimulation, as typically occurs during pathogenesis events in the NP, and can be used to estimate the quantity of neutrophils in a sample even in the presence of nasal epithelial cells, a cell type that is usually present in NP wash samples along with neutrophils. The method described here is of value for the study of neutrophil numbers in nasal wash, a minimally-invasive sample collection procedure, and should facilitate research involving the human upper respiratory tract.

2. Materials and Methods

2.1. Neutrophil isolation:

For development of the method, whole blood was collected from healthy adult donors at Rochester General Hospital in accordance with an IRB-approved protocol (approval number 1141B). Neutrophils were isolated from whole blood using an EasySep Human Neutrophil Enrichment Kit (StemCell Technologies) according to the manufacturer's instructions.

2.2. Cell stimulation:

All experiments were performed using enriched neutrophils serially diluted in RPMI+10% FBS+1% Penicillin/Streptomycin. First, enriched neutrophils from each donor (serially diluted to a concentration of 10^3 to 10^6 per mL) were stimulated with or without heat-killed *Streptococcus pneumoniae* (Spn; 10^8 CFU equivalents for MOI ranging from 10^2-10^5) for two hours before quantification. In separate experiments, enriched neutrophils from several donors were mixed with varying numbers of nasal epithelial cells (D562) for two hours, without bacterial stimulation before quantification. Experiments were conducted at 37°C and 5% CO_2.

2.3. Nasal wash and flow cytometry:

Fresh nasal wash samples were collected from children aged 6-48 months by instillation and immediate aspiration of 3mL of phosphate buffered saline as approved by the IRB of Rochester General Hospital (approval number 1141B). The samples were obtained from children experiencing a viral upper respiratory infection or middle ear infection. Samples from healthy children did not contain enough cells to permit analysis. 200μL of whole NP wash was used for flow cytometry with analysis completed within 3 to 5 hours of collection (the remainder was preserved in TRI Reagent as described below). Samples were stained for 20 minutes at room temperature with antibodies against human CD16 and CD66b (BioLegend) and analyzed using FlowJo version 10 (Tree Star). The same samples were tested by RT-qPCR after freezing (see below).

2.4. RNA isolation and RT-qPCR:

NP wash samples were mixed with TRI Reagent (Sigma), followed by RNA extraction. Briefly, chloroform was added to each sample (0.2x starting volume) for 3 minutes, followed by 15 min centrifugation at 12,000g at 4°C. Next, isopropanol was added to the aqueous phase of each sample (0.5x starting volume) for 10 minutes, followed by 10 min centrifugation at 12,000g at room temperature. The isopropanol supernatant was discarded, and 1x starting volume of 75% ethanol was added to each sample, followed by a final 10 min centrifugation at 12,000g. RNA samples were resuspended in 30μL UltraPure nuclease-free water (ThermoFisher), and purity and concentration were measured by NanoDrop. Due to variability in RNA concentrations, the maximum volume (14.2μL) was used for cDNA synthesis. cDNA was prepared using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA was diluted 1:5 in nuclease-free water, and 5μL of the diluted cDNA was used to perform qPCR with a SYBR Select master mix (Applied Biosystems) targeting *ITGAM* (CD11b), *FCGR3A* (CD16), *LTBR* (CD18), *SELL* (CD62L), and *CEACAM8* (CD66b), with *GAPDH* and *18S* rRNA used as reference genes (primer sequences in Table 1). Data are expressed as raw critical threshold (CT) values or fold change calculated by the ddCT method as indicated.

2.5. Statistics:

Correlations and regression coefficients were calculated using the lm function in base R version 3.1.1 (www.r-project.org). A 2-tailed Student's *t* test was used to compare sample populations; bars in figures depict mean±SEM.

3. Results

3.1. mRNA encoding CD16, CD18, and CD62L are stably expressed and not significantly altered by bacterial stimulation:

Prior publications on cell surface markers commonly used to identify neutrophils by flow cytometry were considered to select promising gene targets for amplification. The most prominent neutrophil markers include Macrophage-1 antigen, a complement receptor composed of CD11b (encoded by *ITGAM*) and CD18 (encoded by *LTBR*); the Fc receptor CD16 (encoded by *FCGR3A*); the selectin CD62L (encoded by *SELL*); and the *Neisseria*

receptor CD66b (encoded by *CEACAM8)* (Futosi et al., 2013; Youinou et al., 2002). We therefore performed qPCR for these genes in samples of neutrophils isolated from whole blood. *ITGAM* and *CEACAM8* were not reliably detectable by qPCR in samples containing $<10^5$ neutrophils (data not shown), but *FCGR3A, LTBR,* and *SELL* were readily amplified, with CT values for each gene below 38 (the manufacturer's reported limit of detection in qPCR) in samples containing as few as 10^4 neutrophils (Fig. 1A).

Since the target samples for this method would be NP wash and such samples typically include bacteria, especially Spn, we sought to determine the effect of Spn on neutrophil quantification with the qPCR method. We found that transcription of mRNA encoding CD16, CD18, and CD62L was not significantly altered by HK-Spn stimulation, and the mean of all three is more resilient still, with only minimal variations in transcription within and between treatment groups (Fig. 1B). Transcription of *CXCL8,* which is expressed by neutrophils in response to stimuli (Chiewchengchol et al., 2016; Fujishima et al., 1993), was significantly upregulated by HK-Spn stimulation of purified neutrophils (Fig. 1C), demonstrating responsiveness of the samples to stimulation.

3.2. Mean CT of mRNA encoding CD16, CD18, and CD62L closely correlates with neutrophil count:

Having established that transcription of *FCGR3A, LTBR,* and *SELL* was reliably detectable and not significantly affected by stimulation with HK-Spn, we tested the correlation between the mean CT value of *FCGR3A*, *LTBR*, and *SELL* (hereafter "mean neutrophil CT") and the neutrophil content in each sample of neutrophils isolated from blood. Since neither the reference genes (*GAPDH* and *18S*) nor the neutrophil-specific genes (*FCGR3A*, *LTBR*, and *SELL*) were significantly affected by bacterial stimulation (Fig. 1B), we proceeded under the assumption that the chief influence on their amplification would be the neutrophil count. We found a highly significant correlation between the log-transformed neutrophil content of a sample and the mean neutrophil CT (Fig. 2), which could be expressed as the following:

$$y = m \log_2 x + b$$

where x = the neutrophil content, y = the mean CT value of mRNA encoding CD16, CD18, and CD62L, $m = -1.54 \pm 0.10$, and $b = 54.20 \pm 1.73$. Rearranging this to solve for the neutrophil content x yields:

$$x = 2^{\frac{y-b}{m}} \quad \text{Equation 1:}$$

3.3. Specificity of mean neutrophil CT in the presence of other cell types:

Pure populations of neutrophils are not usually encountered *in vivo*; instead neutrophils are usually encountered as part of a heterogenous mixture of leukocytes and epithelial cells (Farrera and Fadeel, 2013; Heron et al., 2012; Nicu and Loos, 2016; Werner et al., 2011). Since these other cell types also express Fc receptors, pathogen recognition receptors, and/or selectins (Golebski et al., 2015; van Tongeren et al., 2015), we sought to determine whether

the relationship described in Equation 1 could accurately measure neutrophil content in a heterogenous sample containing nasal epithelial cells. To test this, we adjusted our *in vitro* model by combining varying numbers of neutrophils enriched from healthy adult blood with varying numbers of human nasal epithelial cells (D562 cells), a cell line derived from human nasopharyngeal epithelium commonly used as a model in *in vitro* studies of respiratory immunity (Bertuccini et al., 2004; Khan and Pichichero, 2012). In the presence of D562 cells, the relationship between neutrophil count and mean neutrophil CT shifted (Fig. 3A), indicating that these cells possess contain appreciable quantities of *FCGR3A, ITBR,* and *SELL* mRNA.

We determined that a mathematical adjustment for the CT values could correct for the influence of D562 cells. In enriched-neutrophil samples, the mean dCT of *FCGR3A, LTBR,* and *SELL* (after subtracting the mean CT of *GAPDH* and *18S*) was 7.81±0.29. When D562 cells were added to the cultures, dCT values tended to be greater. Thus, subtracting 7.81 from the observed mean dCT of *FCGR3A, LTBR,* and *SELL* in samples containing both D562 cells and neutrophils approximates the magnitude of the D562 influence. For example, the mean dCT of *FCGR3A, LTBR,* and *SELL* in a sample of 1.4×10^5 neutrophils together with 5.2×10^5 D562 cells was 12.61. Subtracting 7.81 from this number returned a correction factor of 4.80. This correction factor could then be added to the mean CT of *FCGR3A, LTBR,* and *SELL* to yield a corrected mean neutrophil CT which closely follows the pattern established by pure neutrophil populations (Fig. 3B). When applied to Equation 1, the corrected mean neutrophil CT returned a more accurate approximation of the neutrophil content in a sample containing both neutrophils and human nasal epithelial cells (Fig. 3C).

3.4. Neutrophil content of nasal wash samples:

We next performed flow cytometry on nasal wash samples obtained from young children experiencing viral upper respiratory infections or acute otitis media to assess the neutrophil content of *in vivo* samples. In eleven samples, we found that 79±5% of the cell content in nasal wash was made up of neutrophils (defined as cells expressing both CD16 and CD66b) (Fig. 4A, B). The remainder of each sample was frozen in TRI reagent and then used to validate the PCR method described above. Since neutrophils were the predominant cell type in these samples, the correction factor was relatively small (mean −0.75±0.26). Neutrophil counts estimated by flow cytometry were highly correlated with predictions by qPCR (Fig. 4C). The qPCR method tended to predict more neutrophils (mean of 2.25±0.70-fold higher) than flow cytometry. The ranks of neutrophil counts generated by both methods correlated closely (Fig. 4D), such that samples with a higher neutrophil count predicted by PCR also had a higher neutrophil count estimated by flow cytometry.

4. Discussion

We describe a novel quantitative method for estimating the neutrophil content of frozen samples of nasal washes from the nasopharynx of human subjects. The human nasopharynx is a clinically significant polymicrobial niche involved in upper and lower respiratory infections (Chonmaitree et al., 2016; Gill et al., 2008; Teo et al., 2015; Tomlinson et al., 2014). Immunity in the nasopharynx is carefully orchestrated to maintain a homeostatic

balance between excessive tolerance and immune pathology (Chao et al., 2015; Phipps et al., 2007; Ward et al., 2009). There is no molecular method currently described that can accurately determine the quantitative recruitment of neutrophils to the nasopharynx.

After RNA extraction and cDNA synthesis, the mean CT values of *FCGR3A, LTBR,* and *SELL* mRNA (the genes encoding CD16, CD18, and CD62L) determined by qPCR can be used to estimate the quantity of neutrophils in a sample with a limit of detection around 10^4 neutrophils. Though the stability of *GAPDH* transcription has been questioned in neutrophil gene expression studies (Zhang et al., 2005), we observed no significant effects on its expression by HK-Spn stimulation. The simultaneous use of *GADPH* and *18S* supports our interpretation that both genes are stable reference genes. The structural genes we tested did not show significant variability in response to stimulation by bacterial components commonly encountered in the nasopharynx, suggesting that the results will not be strongly affected by the exact inflammatory status of a subject.

The qPCR measurement could be mathematically adjusted to provide accurate results in the presence of epithelial cells (even when neutrophils make up only 6% of the cells in a sample). Estimation of neutrophil content in heterogenous samples required the correction, since calculation based on the uncorrected mean neutrophil CT tended to overestimate the neutrophil content by a clinically relevant margin. Since epithelial cells only minimally express the genes used to calculate the mean neutrophil CT, their contribution could be readily identified. Since samples of nasal wash are predominantly composed of neutrophils during illness, our method can be used to estimate the number of neutrophils present in these samples. Similarly, burn injuries are also rich in neutrophils and epithelial cells, making burned skin samples a good candidate for examination by this method. However, other leukocytes highly express CD16, CD18, and CD62L, and are abundant in the lungs during inflammation (Ghoneim et al., 2013; Heron et al., 2012), so this method is unlikely to be accurate with BALF. A recent study of BALF in various human diseases found that neutrophils typically made up only 6% of BALF cell content, while alveolar macrophages and lymphocytes constituted 86% (Wojtan et al., 2016). We were unable to assess this independently as we do not have access to human BALF samples.

A great advantage to this method is that it does not require live cells and can be performed on preserved clinical samples. Flow cytometric or histological staining must be performed with live cells immediately after collection. RNA extraction for reverse transcription by qPCR, in contrast, can be performed at a later date, requiring only that cells be preserved in a lysis buffer such as TRI reagent, after which they can be stored at −80°C for months to years. This greatly facilitates collection and batch analysis of samples. While the PCR-based counts do not perfectly reflect those derived from flow cytometric analysis of the same samples, the estimates of neutrophil content correlated closely with one another across both methods, and were within an average of 2-3 fold. Furthermore, nasal wash samples are a highly heterogenous mixture of saline solution and mucous secretions, such that the flow cytometry estimates of neutrophil counts are themselves subject to considerable variability. Overall, the PCR estimates can be compared favorably to methods such as bacterial quantification, which express counts of colony-forming units with respect to powers of 10.

Estimation of neutrophil content in a sample will inform assessments of cytokine and chemokine production. During inflammation, neutrophils are recruited to the site of infections in response to elevated secretion of chemokines such as IL-8 and leukotrienes (Nicu and Loos, 2016) during the early, pro-inflammatory phase of an immune reaction. The later, resolving phases are characterized by the apoptosis and removal of neutrophils and increased production of anti-inflammatory factors such as IL-10 and TGFβ (Morris et al., 2015, 2014; Zhou et al., 2008). Thus, the ability to measure neutrophil content in a sample will improve understanding of the progression of inflammation during infection and injury.

5. Conclusion

We describe a novel procedure for quantifying neutrophils in frozen nasopharyngeal samples also containing epithelial cells. We have validated the assumptions underlying this measurement in nasal wash samples, and propose that it may be applicable to other types of samples in which neutrophils constitute the most abundant leukocyte population.

Acknowledgments

Funding: This work was supported by the National Institute on Deafness and Other Communication Disorders (R01 DC008671).

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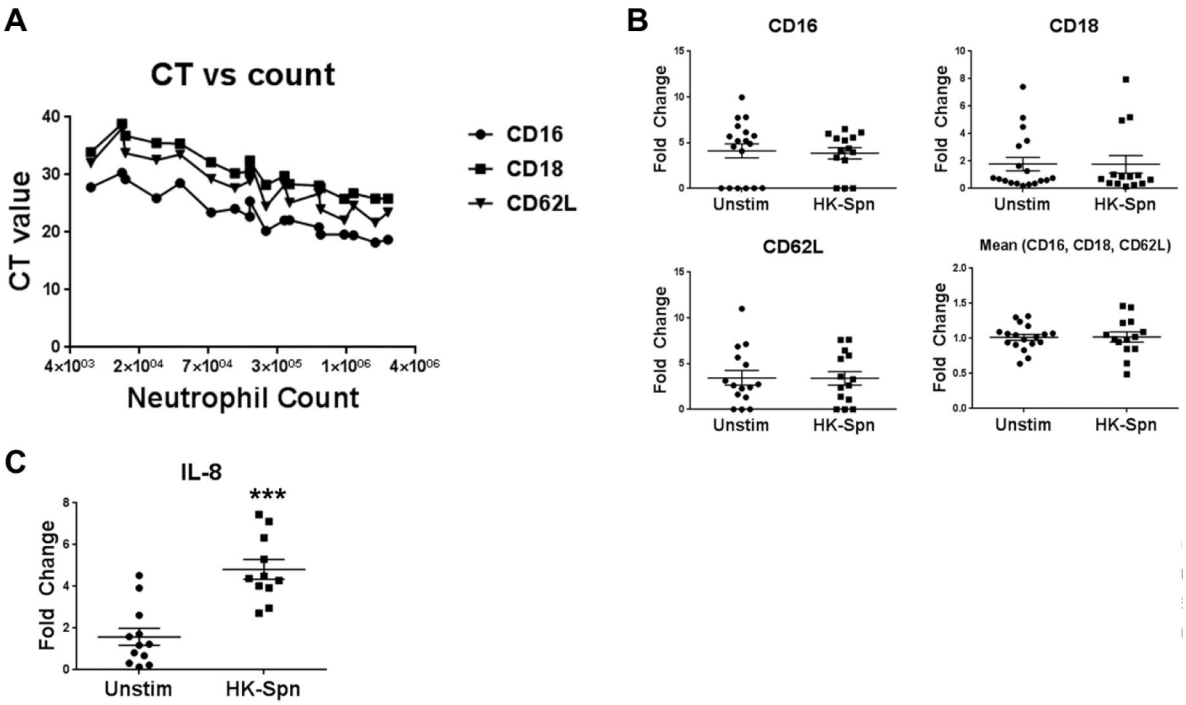

Figure 1.

A) CD16, CD18, and CD62L are reliably detectable in samples containing as few as 10^4 neutrophils. B) Transcription of CD16, CD18, and CD62L is not significantly altered by stimulation with HK-Spn, and the mean expression of all 3 genes shows increased stability. C) IL-8 transcription is significantly increased by HK-Spn ($p<0.0001$).

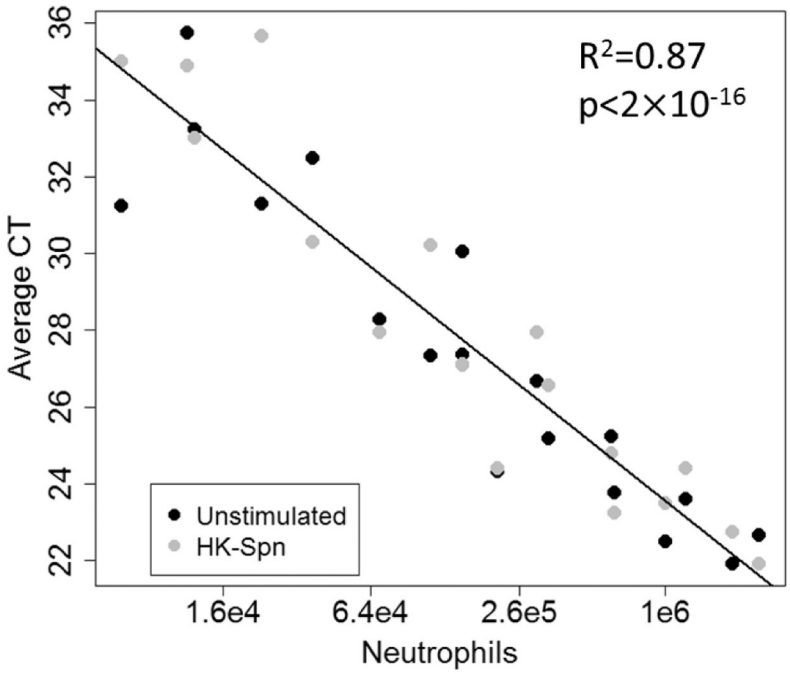

Figure 2.

Average CT value of CD16, CD18, and CD62L can be expressed as a function of log-transformed neutrophil count in a sample isolated from healthy adult blood, and is not strongly affected by 2 hour stimulation with heat-killed Spn. Results are from 3 donors in 3 independent experiments with 6 dilutions each; fitted line depicted in black.

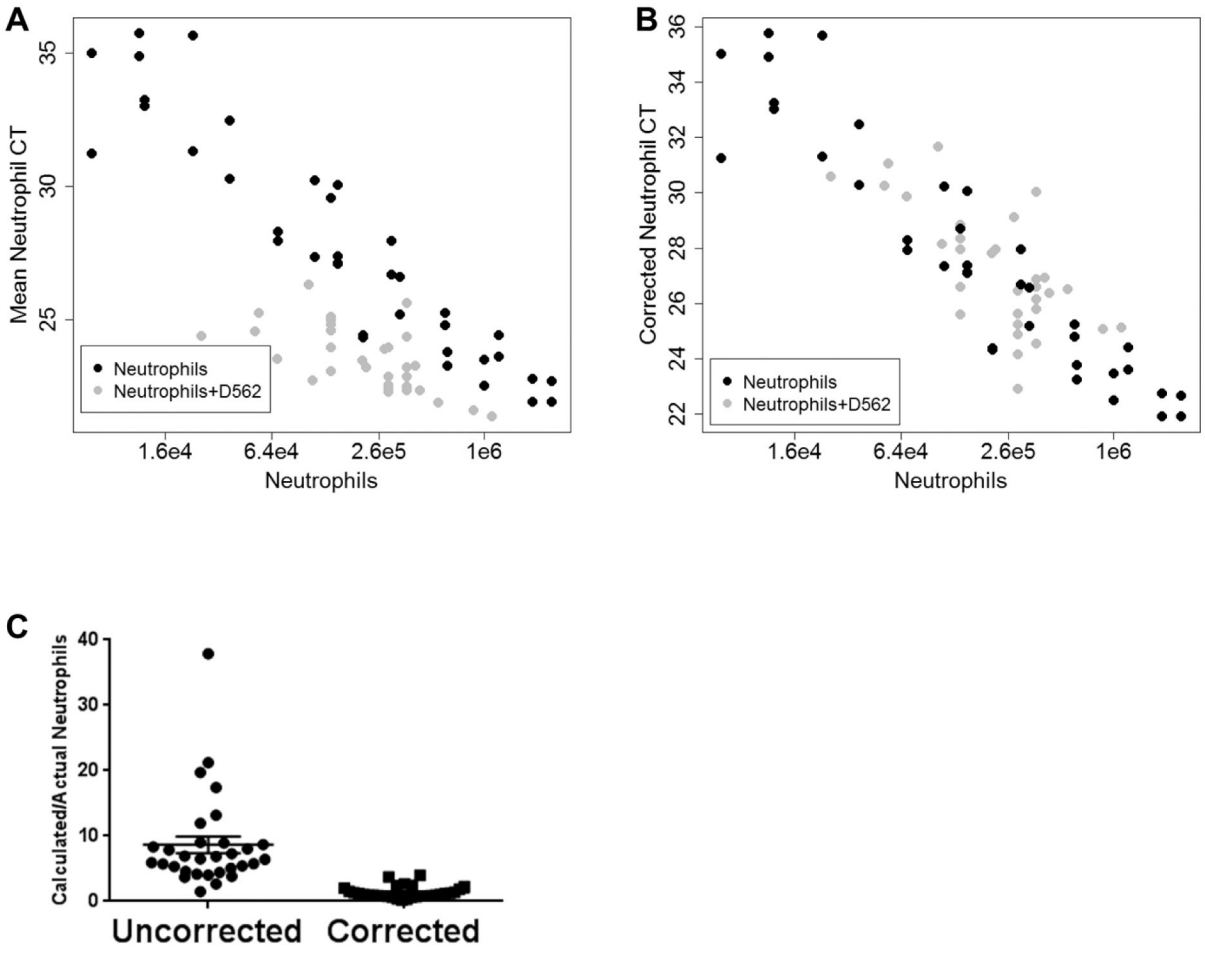

Figure 3.

Specificity of mean neutrophil CT in the presence of varying numbers of D562 respiratory epithelial cells. A) Mean neutrophil CT of heterogenous cultures is shifted. B) Adjusting the mean neutrophil CT according to a correction factor derived from the mean dCT of CD16, CD18, and CD62L relative to GAPDH and 18S corrects for the shift introduced by D562 cells. C) Accuracy of calculated neutrophil content in heterogenous samples using corrected and uncorrected CT values (Uncorrected mean accuracy = 8.40±1.27; Corrected mean accuracy = 1.33±0.16).

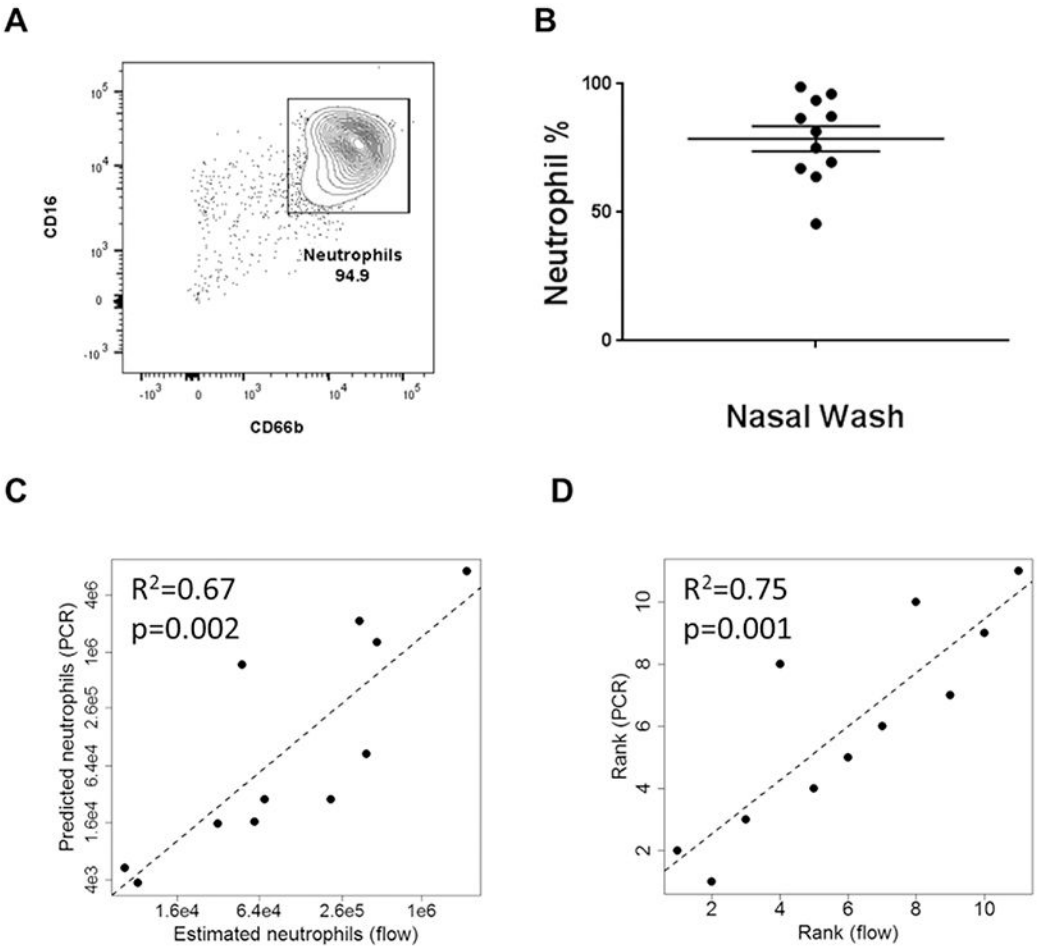

Figure 4.

Assay performance in human samples. A) Representative flow cytometry plot of neutrophils present in a sample of total, unprocessed nasal wash. B) Neutrophil content of 11 nasal wash samples. C) Accuracy of PCR prediction compared to neutrophil count estimated by flow cytometry. D) Comparisons of ranked neutrophil counts estimated by PCR and flow cytometry.

Table 1.

Primer sequences used in these experiments. All primers were verified to target mature RNA sequences and exclude genomic DNA by NCBI's BLAST tool. All melt curves for qPCR results had only one peak.

Gene name		Primer Sequence (5'-3')
GAPDH	F	ACGGATTTGGTCGTATTGG
	R	GGAAGATGGTGATGGGATTT
18S	F	GAGACTCTGGCATGCTAACTAG
	R	GGACATCTAAGGGCATCACAG
FCGR3A (CD16)	F	TCGAGCTACTTCATTGACGC
	R	GATATGGACTTCTAGCTGCACC
LTBR (CD18)	F	CTGGAACTACCTGACCATCT
	R	GTTACCCTTCCCAACTTCATC
SELL (CD62L)	F	CTAGAGAAGGACCAAGCAAAG
	R	CCGGATTCCTATCCAGTAGTA
CXCL8 (IL-8)	F	GCAGCCTTCCTGATTTCT
	R	ACTTCTCCACAACCCTCT