Noninvasive Determination of CMV Serostatus From Dried Buccal Swab Samples: Assay Development, Validation, and Application to 1.2 Million Samples

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Background. Buccal swab sampling constitutes an attractive noninvasive alternative to blood drawings for antibody serostatus assays. Here we describe a method to determine the cytomegalovirus immunoglobulin G (CMV IgG) serostatus from dried buccal swab samples.

Methods. Upon solubilization, CMV IgG is determined by an ELISA assay specifically adapted to cope with low IgG concentrations. The derived CMV titer is normalized against the total protein concentration to adjust for incorrectly or less efficiently sampled buccal swabs. Assay parameters were optimized on a set of 713 samples.

Results. Validation with 1784 samples revealed distinct results for > 80% of samples with 98.6% specificity and 99.1% sensitivity. Based on the analysis of 1.2 million samples we derived age- and sex-stratified CMV prevalence statistics for Germany, Poland, United Kingdom, and Chile. To confirm accuracy of the assay in routine operation, the CMV status of 6518 donors was reassessed by independent laboratories based on conventional blood samples revealing 96.9% specificity and 97.4% sensitivity.

Conclusions. The assay accurately delivers the CMV IgG serostatus from dried buccal swab samples for > 80% of the participants. Thereby it provides a noninvasive alternative to plasma-based CMV monitoring for nondiagnostic purposes such as hematopoietic stem cell transplantation donor screening or population studies.

Keywords. CMV; buccal swab; ELISA; seroprevalence; swab; IgG.

Blood is the preferred source to determine the immune status for diagnostic purposes. Appropriate standards have been established to minimize risks associated with blood collection for patients and caregivers. However, the invasive character of blood collection remains a hurdle for analysis of the immune status outside the classical clinical applications like in epidemiological studies. An alternative to blood-based sampling is the use of oral fluids for the detection of specific antibodies [1–3]. Saliva has been collected with dedicated sampling devices for the detection of antibodies [4]. Often a stabilizer is added to prevent degradation of the antibodies during storage or shipment [5]. Alternatively, point-of-care diagnostic tests circumventing the need for preservation have been developed for diseases with major health care implications like human immunodeficiency virus (HIV) or hepatitis C virus [6–8].

The main focus of our laboratory lies in human leukocyte antigen (HLA) genotyping of potential donors for hematopoietic stem cell transplantation [9]. Worldwide, DKMS collects more than a million such samples each year [10]. To facilitate fast selection of the optimal donors for patients in need of a stem cell transplantation, we strive to characterize all aspects with relevance for transplantation at the time of donor registration. Albeit secondary to HLA matching, there is evidence that overall survival is significantly increased if the cytomegalovirus (CMV) status is matched [11]. Previously, the CMV status of new donors had been determined using blood samples obtained during registration events. However, the majority of new donors register online and send only a buccal swab sample. Therefore, we decided to establish an assay to determine the CMV immunoglobulin G (IgG) status based on swabs. Because the CMV status is nonessential information for donor selection, we decided to accept a total failure rate of up to 25%. We targeted an error rate of < 3% as the CMV status is confirmed based on blood samples before transplantation. Starting from a commercially available plate-based CMV IgG enzyme-linked immunosorbent assay (ELISA), we generated an assay delivering accurate CMV status from dried buccal swab samples fulfilling the abovementioned criteria.

METHODS

Samples

Volunteers from Germany, Poland, United Kingdom, and Chile provided blood (2015–2017) or cheek swab (2018–2019) samples to DKMS for registration as potential hematopoietic stem cell transplantation donors. Samples were collected either at local donor drives or at home after online registration. Donor drives are organized locally by supporter groups (supervised by DKMS employees), usually with some personal connection to a patient in need of a transplantation. These groups use a variety of advertising options to motivate for the one-time collection event. Most donors nowadays register online after becoming aware of the cause by social or classical media. Registration is free of charge; however, new registrants are encouraged to contribute to their registration costs. Only individuals declaring to meet the medical requirements [12] are registered. In Germany more than 10% of the population in the relevant age group have registered [10]. The regional distribution of the cohorts described herein, their proportion of the general population, age, sex, registration mode, and observed CMV seroprevalence are available in Supplementary Data. Volunteers self-specified ancestry during registration. This information was used to partition samples into foreign and native groups. For validation of the assay, 2497 volunteers from Germany provided blood and buccal swab samples at 3 independent donor drives. Samples with equivocal results in the reference values obtained from the blood samples were excluded from further analysis. All subjects gave written informed consent for CMV status determination in accordance with the Declaration of Helsinki.

Determination of Reference Values From Blood Samples

Reference CMV IgG serostatus values were obtained by performing the CE-certified CMV IgG ELISA (EIA-3468; DRG Instruments) according to instructions. A 2 µL sample of plasma obtained from EDTA blood was diluted with 200 µL sample diluent.

Swab-Based CMV Assay

Sampling

Three samples were collected with FLOQSwab hDNA free (Copan Italia). Volunteers were instructed to rub the swab for at least 60 seconds on the inside of the cheek.

Sample Preparation

For extraction of sample material, swabs were shaken for 1 minute in 300 µL phosphate-buffered saline (PBS) buffer (0.01 M phosphate buffer, 0.0027 M KCl, 0.14 M NaCl, pH 7.4 at 25°C; Genaxxon PBS buffer tablets) followed by 30 minutes incubation at room temperature.

ELISA

The CMV IgG assay for blood plasma was adapted for saliva-based samples by DRG Instruments (catalog number SLV-6024). Samples were processed according to manufacturer instructions of the plasma assay with the following modifications: 100 µL of eluate (instead of 2 µL plasma) was diluted with 100 µL sample diluent; temperature of the primary incubation step was reduced from 37°C to 31°C.

Total Protein Assay

The total protein content of the eluate was determined using the Pierce bicinchoninic acid assay (BCA) Protein Assay Kit (catalog number 23 225; Thermo Scientific) according to manufacturer instructions with the following modifications: the assay was performed in 384-well plates at room temperature and the volumes were adjusted to 12.5 µL sample volume and 100 µL work-reagent volume.

CMV assay units were normalized against the protein content to a protein concentration of 1 mg/mL (normalized units). Samples with protein concentrations below 0.2 mg/mL were flagged and excluded from CMV determination (insufficient material).

Quality Control

The following parameters were checked for each CMV assay plate (standards, negative control, and 79 samples) to guard against technical failures: absolute and relative values of the standard curves for both assays; proportion of samples with low protein content; proportion of CMV-positive and -negative samples with country-specific expectation values; and proportion of samples with equivocal results. Sample groups not passing all quality control criteria were subjected to repeat analysis.

Instrumentation

We used 2 identical fully integrated robot platforms supplied by Analytik Jena to perform the ELISA and BCA total protein assay (Supplementary Table 1).

RESULTS

Assay Setup

Proof-of-concept studies indicated that CMV antibodies can be detected by ELISA in buccal swab samples collected from CMV-positive subjects. Even drying the flocked swabs after collection did not interfere with detection of the antibodies. However, the concentration of functional CMV IgG antibodies in the eluate was more than 100-fold lower than in plasma. Therefore, in contrast to the plasma CMV assay, which starts with a 1:100 dilution of plasma, we used 1:2 diluted elution solution in the assay. In addition, ELISA assay components were adjusted to increase sensitivity.

We performed limited testing on the effects of sampling on CMV readout: CMV signal intensity increased with the time of swabbing levelling out at about 30 seconds (data not shown). We advised donors to swab thoroughly between cheeks and gums for 1 minute to minimize assay variation from random swabbing time. Wetting the swab on the tongue before sampling did not improve the assay but rather slightly reduced assay readout

(data not shown). We found no major influence of the time of sampling between 8 AM and 5 PM (data not shown).

Because inadequate sampling may reduce the CMV assay signal, we performed a total protein assay to prevent false-negative results. For eluates with insufficient total protein content (< 0.2 mg/mL) no CMV result was reported. Further, the total protein readout was applied to normalize the CMV signal. This improved assay accuracy considerably by both reducing the number of false negatives and false positives (Figure 1 and Supplementary Figure 1).

Parameter Optimization

For parameter optimization and validation, donors at 3 donor drives were asked to provide both a blood sample and 3 buccal swab samples. In total, 2497 donors provided both sample types. We randomly selected a subset of 713 samples of the first 2 donor drives to determine optimal assay parameters. CMV values determined from the blood samples served as reference values.

To determine optimal cutoff values for positive and negative samples we determined the accuracy for 8 ranges of normalized units (Figure 2A) for samples with > 0.2 mg/mL total protein. Most of the samples could be clearly detected as positive or negative. However, between 2 and 40 normalized units positive and negative samples were overlapping to varying degrees. Targeting an overall assay success rate of 80% at below 3% error rate, we set the following thresholds: negative, < 8 normalized units; and positive, > 20 normalized units; 8–20 normalized units were defined as equivocal results.

We verified the total protein cutoff parameter of 0.2 mg/mL by determining the accuracy for 9 ranges of total protein content (Figure 2B). Samples with a protein content between 0 and 0.2 mg/mL showed error rates of 18% (0–0.1 mg/mL) and 5% (0.1–0.2 mg/mL). With protein contents higher than 0.2 mg/mL the error rates dropped below 5%. This confirmed the cutoff parameter of 0.2 mg/mL.

Assay Sensitivity and Specificity

Using the parameters determined above, 3 independent sample sets were analyzed to evaluate the robustness of our assay. In total this amounted to 1784 samples excluding the samples used for parameter
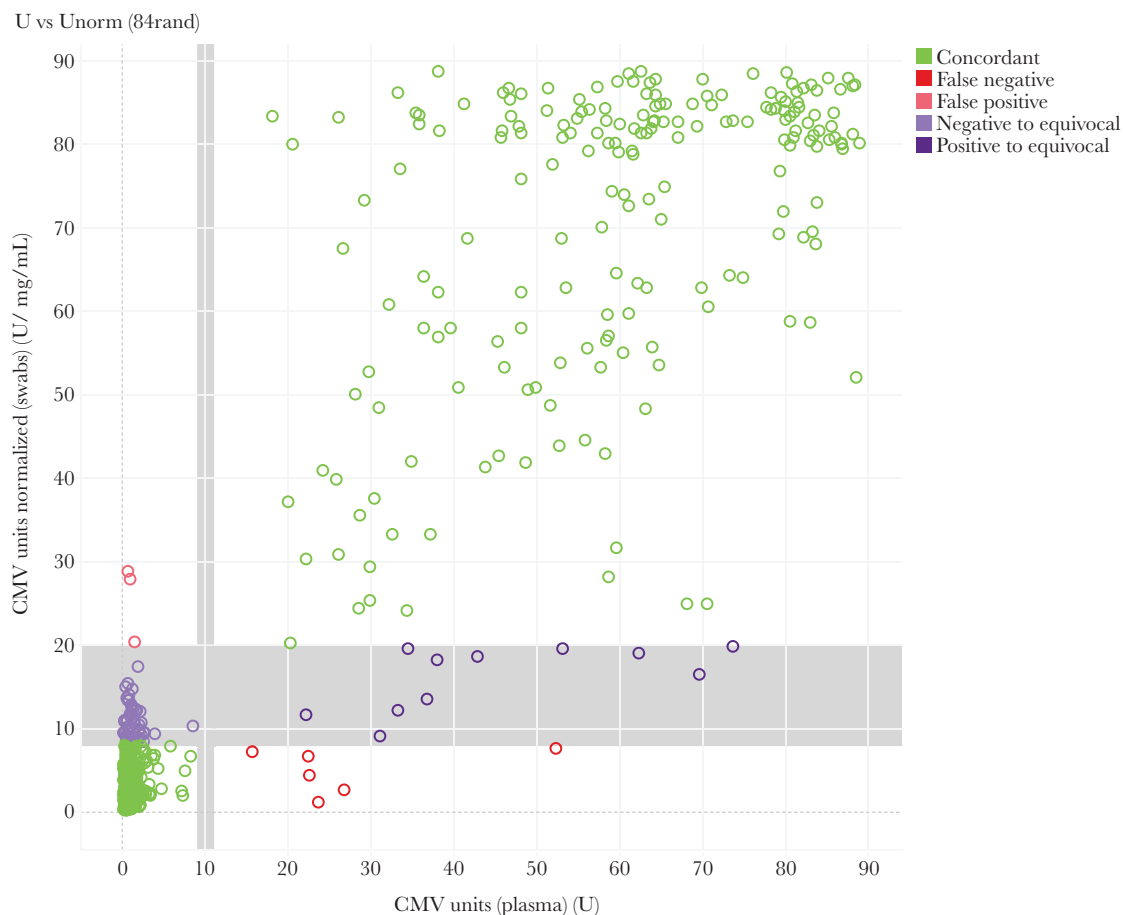

Figure 1. Normalization against total protein content improves assay accuracy. Correlation of cytomegalovirus (CMV) units as determined from plasma (x-axis) with swab-based units (y-axis) after normalization for total protein concentration. Samples with total protein concentration of <0.2 mg/mL were excluded. Normalization for total protein concentration reduced the proportion of false negatives and false positives (lower right/upper left quadrant).
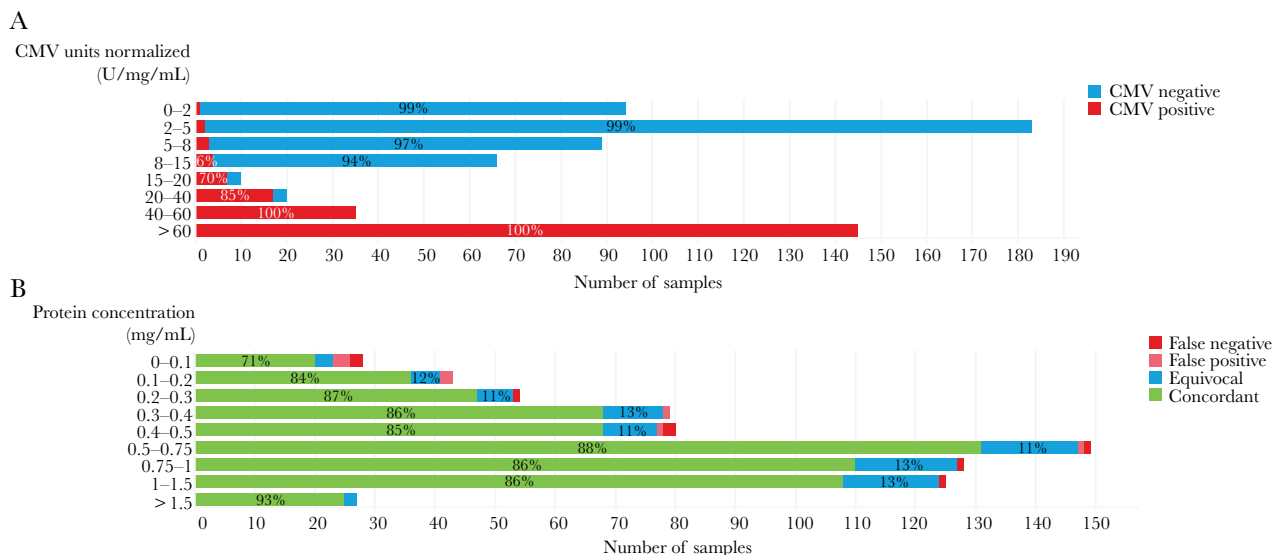
Figure 2. Parameter optimization based on a set of 713 randomly selected samples. *A*, Positive/negative cutoff values: samples are grouped in bins of normalized cytomegalovirus (CMV) units. Positive/negative classification is based on plasma CMV assay. *B*, Minimal total protein content: samples are grouped in bins of total protein content and classified based on previously determined cutoff values.

optimization (Table 1). Assay sensitivity and specificity was determined to be 99.1% ± SD 0.8% and 98.6% ± SD 0.4%, respectively. In all 3 sample sets sensitivity and specificity reached at least 98%.

Prolonged Swab Storage Times

In order to assess the effect of prolonged storage times, 3 swabs from each donor of the third donor drive were analyzed at 3

time points. The first measurement was performed immediately after swab arrival. The second measurement was performed after 1 month and the third after 2 months of storage of the dried swabs at room temperature.

The results show a slight increase in error rates over time from 1.2% to 3.0% (Supplementary Table 2). Plotting the normalized CMV units based on swabs versus the blood-based

Table 1. Parameter Determination and Validation Based on 3 Sample Sets With 2497 Samples in Total

Samples Total, No. (%)	Samples Without Result, No. (%)			Samples With Result, No. (%)							Specificity, %	Sensitivity, %
	Total	Low Protein Content	Equivocal	Total	Correct			False				
					Total	Positive	Negative	Positive	Negative			
Parameter determination												
713	147	71	76	566	557	197	360	3	6			
(100)	(20.62)	(9.96)	(10.66)	(79.38)								
					(100)	(98.41)	(35.37)	(64.63)	(0.53)	(1.06)	99.2	97.0
Donor drive 1												
496	123	66	57	373	369	152	217	4	0			
(100)	(24.80)	(13.31)	(11.49)	(75.2)								
					(100)	(98.93)	(41.19)	(58.81)	(1.07)	(0.00)	98.2	100.0
Donor drive 2												
886	163	105	58	723	713	174	539	8	2			
(100)	(18.40)	(11.85)	(6.55)	(81.6)								
					(100)	(98.62)	(24.40)	(75.60)	(1.11)	(0.28)	98.5	98.9
Donor drive 3												
402	71	38	33	331	327	105	222	2	2			
(100)	(17.66)	(9.45)	(8.21)	(82.34)								
					(100)	(98.79)	(32.11)	(67.89)	(0.60)	(0.60)	99.1	98.1
Total without parameter group												
1784	357	209	148	1427	1409	431	978	14	4			
(100)	(20.01)	(11.72)	(8.30)	(79.99)								
					(100)	(98.74)	(30.59)	(69.41)	(0.98)	(0.28)	98.6	99.1

Table 2. Performance Characteristics of Routine Samples Analyzed With the Swab Assay Determined Against Clinical CMV Results (Plasma Based)

Country	Total	Total	Positive	Negative	Positive	Negative	Specificity, %	Sensitivity, %
		Samples With Results, No. (%)						
		Correct			**False**			
Germany	5196 (100)	5056 (97.3)	1982 (38.1)	3074 (59.2)	88 (1.7)	52 (1.0)	97.2	97.4
Poland	870 (100)	836 (96.1)	652 (74.9)	184 (21.1)	15 (1.7)	19 (2.2)	92.5	97.2
United Kingdom	292 (100)	285 (97.6)	140 (47.9)	145 (49.7)	4 (1.4)	3 (1.0)	97.3	97.9
Total	6358 (100)	6177 (97.2)	2774 (43.6)	3403 (53.5)	107 (1.7)	74 (1.2)	97.0	97.4

CMV units indicates that with prolonged storage at room temperature the concentration of detectable antibodies was slightly decreased (Supplementary Figure 2). This does not interfere with the analysis of high-titer samples. However, with time, intermediate-titer samples are increasingly generating too low signals resulting in equivocal or false-negative calls and thereby reducing specificity. In contrast, the drop in sensitivity observed in the 1-month sample set seems not to mark a general trend.

Assessing Routine Accuracy

Albeit consisting of a large number of samples, the above validation data is based on 3 donor drives only. To evaluate if these results can be extrapolated to everyday routine operation, we analyzed the feedback from confirmatory typing. Before hematopoietic stem cell donation, the HLA genotype and CMV status of the potential donor are confirmed by an independent laboratory. As the selection of donors is primarily based on matching of HLA alleles, the selection can be considered to constitute a random subset of all samples analyzed with the swab-based CMV assay. This includes samples from different countries as well as various kinds of donor drives. In addition, it includes a large proportion of samples collected at home without supervision (online registrations).

In total, 6358 donors with swab-based CMV status have been selected for confirmatory typing and analyzed by independent laboratories based on fresh blood samples so far (Table 2). Swab-based CMV results were confirmed for 6177 (97.2%) samples. In total, 181 discrepancies were observed including 74 (1.2%) false negatives and 107 (1.7%) false positives. While slightly higher than during validation (1.3%), this error rate of 2.8% is appropriate for our intended application. In addition, some of the false-negative results might be due to seroconversion between registration and confirmatory typing.

Population Statistics

Between March 2018 and June 2019, we determined the CMV status for more than 1.2 million samples; 67.8% of the samples were from Germany, 21.1% from Poland, 9.6% from the United Kingdom, and 1.5% from Chile. Overall, 35% of the samples were CMV positive, 53% CMV negative, and 12% equivocal. To estimate CMV prevalences in the populations, we attributed the equivocal calls to positive or negative according to the results of the samples retested clinically (see previous

section and Supplementary Table 3). The countries showed major differences in CMV prevalence: 30% of all samples from Germany were CMV positive, followed by 32% from the United Kingdom, and above 60% of samples from Poland and Chile. As expected, CMV prevalence increased with age (Figure 3). In the Polish sample, the CMV prevalence increase was constant (0.7 percentage points/year) over the complete age range from 17 to 50 years. In contrast, CMV prevalence of donors from the United Kingdom and Germany increased only until the age of 35 (Germany 1.1 and United Kingdom 1.2 percentage points/year). Both female and male donors showed the same trend in all countries. On average, women had a 9 percentage points higher CMV prevalence rate than men. Interestingly, despite the major difference in prevalence between Poland/Chile and Germany/United Kingdom, the gender gap was of similar size in all countries and age groups (Supplemental Figure 3).

Finally, we compared these data to CMV prevalence in Germany and Poland determined between 2015 and 2017 based on 226 000 blood samples analyzed in our laboratory (Figure 3). For the German cohort the results were in good agreement with the plasma-based results deviating mostly ±2 percentage points. In the Polish cohort we observed a systematic shift to lower CMV prevalence in the swab-based data by about 5 percentage points, suggesting that the assay parameters may need readjustments for populations with high CMV prevalence.

All analyses regarding CMV prevalence were performed after excluding donors with self-assessed foreign origin to avoid biases due to minority focused campaigns. Analysis of the complete sample set independent of origin revealed 5–10 percentage points higher CMV prevalence for the German and United Kingdom cohorts while the Polish and Chilean cohorts were unaffected (Supplemental Figure 4).

DISCUSSION

Noninvasive oral sampling for determination of the immune status has great potential. In particular, in nonclinical situations blood collections remain a major hurdle [13, 14]. Based on a limited set of 50 samples, the feasibility of CMV IgG testing based on dried swabs has recently been suggested [15]. We demonstrate here, based on a large validation set of 1784 samples, that CMV serostatus can be derived from dried swabs with good sensitivity and specificity. In addition, after processing more
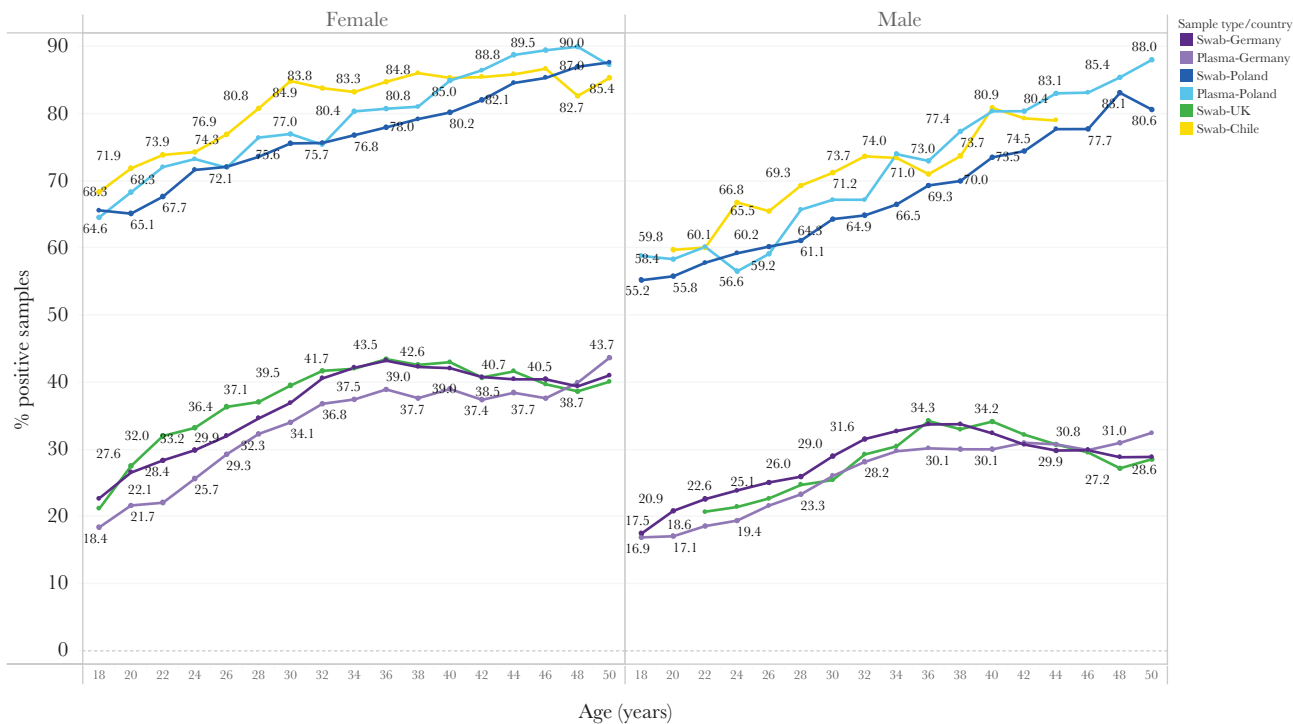
Figure 3. Cytomegalovirus prevalence in 4 countries by sex and age. Donors with self-assessed foreign origin were excluded from the analysis.

than a million samples in the routine workflow since November 2017, we confirmed accuracy of the assay by evaluating the results of a subset of 6358 donors. Those donors were selected for confirmatory typing and the CMV status was determined in independent laboratories based on blood samples applying various certified CMV assays. Compared to the validation data the error rate was slightly elevated to 2.8%. In the Polish subset we even observed an error rate of 3.9%, due to a higher false-negative rate of 2.2% (Table 2). This may indicate that the assay parameters are not ideally fitted for populations with very high seroprevalence. Nevertheless, overall specificity of 97.0% and sensitivity of 97.4% clearly demonstrate the robustness of this screening assay, in particular when taking into account the variability observed with conventional blood-based assays from different vendors [16–19]. Key to this excellent performance was the integration of a secondary assay to control and/or correct for bad or insufficient sampling. By disregarding samples with low total protein content in the swab eluate, false negatives due to insufficient sampling can be prevented. Overall, 2.9% of the samples did not meet this quality criterion. However, swabs from particular donor drives may result in above 20% of noncompliant samples. In such cases, assaying total protein not only prevents an increased error rate but in addition allows for providing direct feedback to the respective coordinators and thereby helps to improve future sampling quality. We have opted for total protein monitoring for practical and economic reasons. However, we assume that normalizing for total IgG

instead could further improve assay specificity. Overall, 15% of samples with sufficient protein concentration yielded equivocal results. Correcting for total IgG might permit a reduction in the proportion of equivocal calls without compromising accuracy. Finally, the consideration of replicate measurements from separately sampled swabs could allow narrowing the equivocal range by controlling for both sampling and analytical variation.

The application of buccal swabs for sample collection enabled us to assay 1 218 000 samples, including 825 000 from Germany, 256 000 from Poland, 117 285 from United Kingdom, and 18 000 from Chile. Therefore, this study constitutes the largest study on CMV seroprevalence for each of the included countries. Overall, the data is in good agreement with previous studies [20–23]. Our data indicates that the seroprevalence in the German and United Kingdom sample, contrary to the expectation, did not increase anymore above the age of 35 years, both for the male and female group. This is further backed up by the independent cohort of 226 000 German samples, analyzed in the years 2015 to 2017 based on blood samples, confirming this finding with very similar values. The findings suggest that the rate of CMV infection in the age group of 35 to 50 years is very low in Germany and the United Kingdom. Unfortunately, that does not improve the situation for the most severe health care implication of CMV, namely the consequences of congenital CMV infection. It was estimated that in Germany alone 700 to 1400 (0.1%–0.2%) infants are born each year with permanent physical sequelae such as mental retardation, sensorineural

hearing loss, and developmental delay [24–26]. Our data indicate that the risk of primary CMV infection for women of childbearing age is increased in Germany and United Kingdom compared to Poland: up to the age of 35 years, we observed in these countries with lower overall seroprevalence a slightly steeper increase in seroprevalence. This manifests in a seroprevalence increase of about 19 percentage points in Germany and United Kingdom versus 12 percentage points in Poland between 18 and 35/36 years of age (Figure 3). This underlines the importance of raising awareness in those countries among women who are pregnant or planning to become pregnant. Even though there is no vaccination or approved treatment to prevent CMV infection, adapted hygienic measures have been demonstrated to strongly reduce primary CMV infections during pregnancy [27, 28].

In conclusion, we demonstrate here that the CMV serostatus can be accurately determined based on dried buccal swab samples. The new workflow has already facilitated analyses of more than a million samples from 4 countries. In particular for Germany, the huge amount of data generated indicates that CMV prevalence may currently be overestimated and more women than previously thought may be at risk for primary congenital CMV infection. While the FLOQSwabs have proven to be adequate sampling tools for CMV serostatus analysis, it remains to be seen if these findings can be directly transferred to immune surveillance monitoring of other infections.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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