Author's response to reviews

Title: Cross-sectional study of cytomegalovirus shedding and immunological markers among seropositive children and their mothers

Authors:

Jennifer D Stowell (hzq8@cdc.gov)
Karen Mask (karen.mask@gmail.com)
Minal Amin (dza9@cdc.gov)
Rebekah Clark (rebekahc413@gmail.com)
Denise Levis (igc1@cdc.gov)
Will Hendley (hla0@cdc.gov)
Tatiana M Lanzieri (uyk4@cdc.gov)
Sheila C Dollard (sgd5@cdc.gov)
Michael J Cannon (mrc7@cdc.gov)

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Author's response to reviews: see over
Stowell et al. response to reviewers (reviewer comments in bold, all line numbers refer to the original submission)

Reviewer 1

This a quite interesting and clever study, though with very low numbers of participants. As there are many studies describing the prevalence of CMV excretion in urines and saliva, especially in the US, within various classes of age, the study is not very original. The main interest is probably to describe the distribution of viral loads in the population per age and thus to better identify the risk per class of age. This link could be enhanced by the follow-up announced as a companion study, and whole results presented together would probably be much more informative and original, because follow-up study are not available in this population.

We acknowledge that the number of participants is limited (N=161) relative to what has been done in previous studies of the prevalence of CMV excretion in urine and saliva. We agree with the reviewer that the main interest relates to other variables, such as the distribution of viral loads by age. We highlighted this and other original aspects of the study in lines 210-219.

Also, we agree with the reviewer about the importance of the follow-up study, but due to space limitations and large amounts of data to report, we chose to describe this study and the follow-up study in separate papers.

The study has some pitfalls well underlined and discussed by the authors. The sample size, that make the results significant only as “trends”, especially for high viral loads risk factors identification is, to our opinion, the only problem. The other points are minor and well discussed by the authors.

Again, we acknowledge the limited sample size, but we would point out that in spite of this limitation a number of the associations were statistically significant (see Table 2), which would go beyond calling them simply “trends”.

The authors mentioned the disparity of sampling methods which impairs comparison of urine and saliva viral loads. (The comparison between urine and saliva by qualitative methods has yet been published). This is true but we underline that direct sampling of urine from children is quite impossible, and so the authors did their best. One way to enhance the comparison could have been to report CMV copies per ng of extracted total nucleic acids from both saliva and urines.

As part of a different study, we attempted to standardize saliva samples to total genomic DNA, as the reviewer suggests, but we found it to be an unreliable approach. The problem was that saliva is loaded with bacterial and epithelial cell DNA. Even with the same input volume, the copies per ng varied person to person more than 10-fold, presumably depending on when the person last ate or drank and perhaps on variation in oral hygiene as well.

So instead, for this study we standardized saliva and urine to each other based on input volume, which was straightforward to calculate (see subsequent response to Reviewer 2).

They also underlined the fact that infectious CMV cannot be assessed by PCR in saliva. We agree with that but the higher viral loads are very likely to be associated with the presence of infectious virus (shown by previous studies of virus detection in urines).
Another possible bias is the recruitment within the local population probably not representative of the whole population (may be due to the way of recruitment?) (high rates of breast milk feeding, socioeconomic conditions, day-care center, are factors collected by the authors and that could have largely influenced the CMV transmission and thus, the prevalence of primary infections in children, but may not reach significance in this small population). However, we can consider that this is a descriptive study from a small special population, and here again, the study will be largely enhanced by the companion paper’s results to see how the virus spreads in the population.

We agree with the limitations about the population and lack of culture results. As the reviewer alluded, we describe them in lines 284-286 and 296-302.

To this purpose the link between the detection of low-avidity anti-CMV antibody and excretion is interesting and reflect the route of acquisition of CMV in young children.

The last point is that the excretion prevalence is very high (100% but 8/8) in the >12 months children population, compared to some previous studies using PCR in saliva, suggesting a recruitment bias towards a high-risk population. This is in agreement with the high prevalence of excretion in mothers showing that CMV circulates actively in this population. This last point, could be interesting to discuss in this paper.

We agree that there is a recruitment bias, but the bias is the result of examining shedding prevalence among children already identified as CMV-seropositive (e.g., the 8/8 children referred to by the reviewer are all CMV seropositive). The high level of excretion in mothers is also due to recruitment bias, since we only tested mothers of children who were CMV-seropositive.

Because we already discussed these issues in the Discussion (lines 238-243) and in Figure legend 2, we did not add any additional discussion.

Finally, this study is interesting and will benefit from comparison with the follow up analysis. It could be therefore be published now as a cross-sectional study and followed by a longitudinal study more interesting in terms of routes and consequences of CMV circulation in the population.

- Discretionary Revisions (which are recommendations for improvement but which the author can choose to ignore)

Figure 1 in which the categories are only based on the authors assumption of the route of transmission is quite subjective, though illustrative. May be withdrawn?

We are not sure what is meant by this comment. We could not identify a reference to a transmission route in the legend to Figure 1.

- Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

- Major Compulsory Revisions (which the author must respond to before a decision on publication can be reached)
The study is probably too preliminary to be published as a full length paper. It could better be presented as a short preliminary study (or feasibility?) before a longitudinal study of CMV transmission in population.

As in our response to an earlier comment, we intend to publish both papers, but this one had a large amount of descriptive data such that we felt it should be a full-length paper. We feel that both papers together will tell a more complete story.

Though the viral load measurements are interesting, the small size of the population does not allow to generalize the conclusions to other populations and the authors should be very cautious before counselling on such preliminary results.

In response to this comment and similar comments from Reviewer 2, we revised the title, Abstract, and Discussion in order to temper some conclusions. In particular, we removed the manuscript's subtitle and reworded the conclusion of the Abstract as follows to discourage overgeneralization of our results:

"Young CMV seropositive children, especially those less than one year-old may present high-risk CMV exposures, especially through their saliva, though further research is needed to see if this finding can be generalized across racial or other demographic strata. Data on shedding among young children will be critical for developing and evaluating prevention messages and behavioral interventions in order to identify effective strategies to prevent CMV transmission."

In addition, to add clarity to our conclusions, we deleted the final paragraph in the Discussion and replaced it with a slightly revised version of an earlier paragraph (lines 271-283):

"Taken together, our findings can be used to inform behavioral prevention messages. To decrease transmission of CMV, the American Academy of Pediatrics (AAP) has advised hand hygiene when caring for children, particularly after changing diapers [49]. Similarly, the American College of Obstetricians and Gynecologists has advised women with young children to use safe-handling techniques after handling diapers or after exposure to respiratory secretions [50]. In addition to this advice, researchers who have conducted CMV behavioral interventions have also advised women to avoid kissing young children on the mouth, to refrain from sharing food, drink, and utensils, and to cleanse toys and other objects that may be exposed to children’s body fluids [12, 14]. Our findings suggest that although handwashing to minimize urine exposures is important (e.g., after diaper changes), behaviors that reduce saliva exposures may be even more important. Saliva appears to have higher CMV viral loads and is more likely to get into the environment through drooling, eating, pacifier use, etc. Urine, by contrast, is usually blocked by diapers. Furthermore, saliva has more opportunities to directly contact the eyes, nose and mouth of a pregnant woman (kissing, sharing food and drinks), whereas, urine contact is typically less direct (diaper-to-hand-to-eye). Because women may not recognize how frequently they come into contact with children’s saliva, it may be important to emphasize behaviors that prevent saliva from directly contacting the mouth or other mucous membranes (e.g., kissing on the mouth or sharing food/drink and utensils)."

Reviewer 2

This cross-sectional study of human cytomegalovirus (HCMV) shedding in seropositive children from a population of educated and relatively affluent mothers/infants provides confirmatory findings consistent with what has been published (often decades ago) in a number studies.
We agree and acknowledged in the Introduction (lines 82-83) that our findings confirm several findings from previous studies, including studies done at the reviewer’s institution (e.g., references 17, 23, 30, 37, 39, 42, 44). Certainly, the measurement of CMV seroprevalences and CMV shedding prevalences is well-plowed ground.

However, as we point out in the Introduction (lines 83-88), many aspects of the biology and epidemiology of CMV infection need a more refined understanding—one that can lead to better prevention and treatment—and that is what we attempted to develop in this study (see further details below).

**Major Issues:**

In addition, a major technical issue with this report limits any definitive interpretation of the findings.

We assume this technical issue refers to the PCR assays, which we address in more detail below.

Finally, the text often overplayed findings in the study. As an example line 205, the results did not approach statistical significance (p=0.06), they were not significant. Likewise, the title is not reflective of the limited definitive data that is in the manuscript.

We modified lines 205-207 to read: “…though the latter association did not achieve conventional levels of statistical significance (Table 2, P=0.06)”

Also, we removed the subtitle of the manuscript (“potential implications for viral transmission”) to avoid overstating the implications of our data.

Statements such as line 169 have no place in the results, “Assuming”, results cannot be based on an assumption but data.

We removed the relevant sentence.

Perhaps one of the most apparent problems with the data in this manuscript is the insensitivity of the PCR assays (saliva 1.6x10^3; urine 1.6x10^4). Such poorly performing assays make any data derived in this study of limited interest and limits the validity of the conclusions drawn by the authors, i.e. line 194 claims differences in rates of elevated viral loads using 10^6 for saliva and 10^5 for urine without taking into account the 10 fold difference in assay sensitivity.

The PCR methods as written do incorrectly give the impression that the PCR assay has poor sensitivity. The low sensitivity is not due to the PCR assay but rather is entirely due to the specimen collection and processing which is now better explained in the Methods section of the manuscript, where we added the following:

“The limits of PCR detection were estimated to be 1,600 copies/mL for saliva and 16,000 copies/mL for urine. These limits are considerably higher than our detection limit for sterile specimens (e.g., blood) collected in clinical settings, which is 70 copies/mL. Two factors led to these higher limits of detection: 1) To avoid false-positives, the PCR assay cutoff was raised five-fold from one copy per reaction (70 copies/ml) to five copies per reaction (350 copies/ ml) because saliva is not sterile and urine was collected in an unsterile manner, and unsterile specimens are more susceptible to trace amounts of contamination from the environment; 2) The methods of specimen collection (i.e., swabs and filter
paper) were necessary to enable in-home collection by mothers, but they resulted in reduced sample volume which raised the limit of detection approximately five-fold for saliva in swabs and 50-fold for urine in filter paper.”

The reviewer is incorrect in concluding that we did not take into account the 10-fold difference in assay sensitivity for saliva and urine. The viral loads were standardized as described above based on the input sample volumes, meaning that the individual values of viral loads for saliva and urine are comparable. However, as we explained in the Discussion (lines 288-294), population shedding prevalences and median viral loads (note: we added the word “median” to clarify) were not directly comparable unless we excluded the saliva results that fell below the limit of PCR detection for urine.

**Overall, this manuscript contains very little if any new information and is riddled with technical issues which drastically decrease any definitive interpretation of the information presented in the manuscript.**

In the first paragraph of the Discussion we listed six new findings from our study (lines 210-219):

“1) more than half of CMV-seropositive healthy young children shed CMV DNA in saliva and/or urine, a much higher shedding rate than that of seropositive healthy adults; 2) among seropositive children, the prevalence of shedding and the magnitude of CMV viral loads tended to be greater among younger children; 3) CMV viral loads were typically higher in children’s saliva and urine than in mothers’ saliva; 4) the highest viral loads (> one million copies/mL) were only found in children’s saliva; 5) low CMV IgG avidity was associated with younger age, CMV shedding, and low CMV IgG titers; and 6) high CMV IgG titers were associated with older age.”

The subsequent six paragraphs (lines 220-270) carefully put these findings into context and cited numerous previous studies to explain how our findings contain new information. In the subsequent paragraph (lines 271-283), which is now the concluding paragraph, we explained why these findings are important for refining CMV behavioral prevention messages.

Because the reviewer did not identify other specific technical issues besides the assay limitations, which we addressed, we made no additional changes in response to this comment.