Author's response to reviews

Title: High frequency of Human Cytomegalovirus DNA in the Liver of Infants with Extrahepatic Neonatal Cholestasis.

Authors:

Adriana MA De Tommaso (amdetommaso@mponet.com.br)
Paula D Andrade (paula@fcm.unicamp.br)
Sandra CB Costa (costa@fcm.unicamp.br)
Cecilia AF Escanhoela (fazzio@fcm.unicamp.br)
Gabriel Hessel (ghessel@fcm.unicamp.br)

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Author's response to reviews: see over
Dear Editorial Board of BMC Infectious Disease,

We thank you for the opportunity to send a revised manuscript for consideration. We have attempted to answer all of the reviewer’s suggestions, as well as improve the overall content of the text.

We would like to thank both Dr. Fischer and Nigro for their insightful review of our work and their excellent suggestions, which will enrich our data and make this manuscript suitable for publication.

Bellow we specify specific comments raised by each reviewer in an individual manner. Answers are in blue font.

**Reviewer: Björn Fischler**

1. “The description of the patients is less thorough than that of the virological methods. For example, how was the diagnosis biliary atresia settled? By liver biopsy? Cholescintigraphy? Intraoperative cholangiogram? Postoperative examination of bile duct remnants? What was the age of the control children?”

   The diagnosis of biliary atresia was made by intraoperative cholangiogram, as indicated now in the main manuscript text, method section, page 3.

   The median age of our control children group was 46 days. We have added this information with appropriate age distribution in the text, method section, page 3.

2. “Table 1 is confusing since it is hard to understand the division into 2 groups (with and without asterisc?!). Are the ALT, GT and DB mean values?”

   In regards to Table 1, we agree that the initial manuscript was confusing due to an unanticipated mistake. The group indicated by the asterisk corresponded to the PCR positive patients. We have now added this information to the table heading.

   The ALT, GGT, and DB values indicated in the table are the median values.
3. “Much work seems to have been put into histological examination. However, some of
the parameters are hard to understand. For example, the exact nature of the cholestasis
intracellular? Canalicular?- is not stated. Cholestasis may occur without cholangitis and
vice versa. Not only ductal proliferation, but also paucity of intrahepatic bile ducts need
to be looked for. Table 2 is of interest but these findings need to compared to those of
CMV negative BA patients.”

In our series the extra hepatic cholestasis was predominantly intracanalicular,
however it was not an aim of this study to use histological variants as a differentiation
factor between HCM positive or negative patients.

We have added Table 3 to the revised manuscript. This shows the histological
characteristics of all HCMV negative patients included in our study.

4. “What was the distinction between mild and moderate chronic active inflammation?”

The distinction of mild and moderate chronic active inflammation depended on
the amount of inflammatory cells; i.e- neutrophils and macrophages. This is the routine
grading system used by our institution’s pathology department.

5. “Clear discrepancies between CMV serology and CMV detection in liver tissue were
noted. However, this does not necessarily mean that the serology had a low
sensitivity/accuracy. The authors need to discuss other possibilities. For example, the
aspect of timing of infection and the duration of IgM antibodies need to be accounted for.
Concomittant CMV testing in the mothers of these children would probably have been
informative too. The traditional gold standard method for diagnosing congenital CMV is
the detection of CMV in the urine before 4 weeks of age. This method seems more
sensitive than CMV-IgM in serum (see for example reference 13). The authors need to
discuss why they have not used CMV isolation in urine, nor PCR detection in blood.”

We agree that the traditional gold standard method for diagnosis of congenital
CMV is viral detection before 4 weeks of age. Unfortunately, ours was a retrospective study and within our institution routine testing of urine for CMV is not a routine test. As so, we do not have information regarding timing of infection in our patient population.

Information of CMV status in our patient’s mothers is also not available. The CMV seroprevalence in an unselected Brazilian population of mothers has been reported at 84.4% (as mentioned in reference 29 of our text).

Since we do not have the above mentioned data, we have added to the text an informative line to this effect.

6. “In the abstract BA is used a short form for biliary atresia, without stating its connotation.”

We have now incorporated this change to the abstract.

7. “Details on the ELISA and PCR methods concerning sensitivity would be clarifying.”

We have used PCR as the gold standard reference for our data analysis. We have indicated this in the result section when describing sensitivity of CMV ELISA serology.

8. “For the PCR detection in liver tissue either fresh material or parafin-embedded fragments were used. Did the authors see any differences in the results between these two groups?”

There were no major differences between either fresh or paraffin-embedded fragments. As so, we have not mentioned this in the text.

9. “It is rightly pointed out that a positive CMV finding might delay further diagnostic procedures in these patients. In fact, this has already been described by Tarr et al, in reference 16. Was there a difference in the age of BA diagnosis between CMV positive and CMV negative patients? “

The median age of BA diagnosis in ELISA positive (IgM+) patients was 105 days (range= 60-187 days) and in ELISA IgM negative patients was 89 days (range= 35-239 days).

10. “Was the study approved by ethics committee?”

The study was approved by the ethics committee of the Faculty of Medical
Sciences, UNICAMP, and all parents of subjects provided informed consent prior to enrolling in the study. We have already specified this information in the method’s section.

Reviewer: GIOVANNI NIGRO

1. “It is necessary to include a Table showing the time at which bioptic and serum samples were taken from infants with positive CMV DNA in the liver. In fact, the median infants’ age at enrolment was ranging between 25 and 239 days. This implies that CMV infection could have acquired perinatally or postnatally. To correlate CMV infection with the development af biliary atresia, it is essential to demonstrate that CMV was acquired prenatally (diagnosis within 3 weeks from birth) or to evidence findings supporting a possible prenatal activity of CMV in the liver like typical histologic changes, high CMV IgG avidity, increasing or persistent IgG titres, decreasing IgM levels. Were bioptic and serum samples obtained at the same time or sera were drawn later? If the sera were obtained after the age of 3 months, this could explain the low prevalence of CMV-IgG antibodies, which are highly frequent in Brazilian mothers.”

   The median time between HCMV serology and liver biopsy was 8 days. We strongly agree with Dr. Nigro that it would be interesting to correlate timing of HCMV infection with the development of biliary atresia. However our study was a retrospective analysis and we do not have data regarding mother’s HCMV serology or the patient’s serology prior to their enrollment in our study. We have specified this information in the text.

   IgG avidity or IgG/IgM titers were not done either.

   Our pathologist did not observe the presence of cytomegalic cells or microabscess in any of the studied cases. This has been mentioned in our text.

2. “Were sensitivity and specificity of PCR and serological assays tested? Specificity is essential both for PCR, since 3 infants had hepatic DNA but negative CMV IgG, and for the immunoassay, since 14 infants had CMV IgM. The specificity of the ELISA capture systems is generally suitable, and false positive IgM antibodies are rarely detected.
However, checking confirmatory tests should be done.”

Sensitivity and specificity of PCR were not tested as part of this study, however our laboratory has an internal routine of testing our nested PCR CMV studies with a high sensitivity or specificity compared to antigenemia. Serology was compared to PCR to determine sensitivity and specificity, which were reported in this study, respectively, as 54% and 61%.

3. “Controls are few. In particular, an adequate number of age-matched controls is needed to establish the significance of CMV seropositivity in the infants with biliary atresia.”

We agree with the reviewer. The number of age matched controls is small. However due to the retrospective nature of our study and the need for biopsies in the control patients were unable to enroll more control subjects. Since none of the 9 controls had a positive CMV PCR result, we consider this an informative set of data.

4. “The conclusions should be changed: although CMV inclusions were not found in the liver, Table 2 shows that infants with DNA had some histologic findings which may be consequent to prenatal CMV infection of the liver (i.e. fibrosis, giant cells, inflammation). To this regard, a comparative Table on the histologic findings between DNA-positive and DNA-negative infants could be helpful.”

Table 3 was added to show the histological characteristics of HCMV negative patients.

5. Abstract: results should be clarified (i.e. the number of infants with positive DNA is not mentioned, while the percentage is indicated);

We agree with the reviewer. This has now been incorporated into the abstract.

6. Serological investigations: ELISA systems were commercial (manufacturer should be indicated) or non-commercial?

ELISA systems were commercial, and this was added to the method section.
7. Conclusions: a possible use of antiviral therapy in the infants with CMV infection, and consequent improvement of the outcome, should be mentioned.

   This has been added to the discussion section.

Sincerely,

Dr. De Tommaso