DETECTING CYTOMEGALOVIRUS IN BREASTMILK
Towards a device for self monitoring risks of postnatal infection

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Abstract: Human cytomegalovirus (HCMV) infection is a major cause of morbidity worldwide especially in newborn infants. While congenital HCMV infection affects 2-5% of preterm newborns, the risk of postnatal infection particularly through breast milk is higher in this population (prevalence about 20%) since more than one mother on two is affected. Congenital and postnatal infection can lead to important clinical complications such as deafness, learning disabilities, and mental retardation during childhood. Neonatologists are squeezed in their clinical practice: either breastfeeding is favored without any milk treatment going on exposure of preterm infants to a potential infection, or milk is systematically treated by freezing or pasteurization but with deprivation of non-at-risk infants from the benefits of fresh milk. In this position paper, we propose a possible solution to differentiate milk with risk of HCMV contamination from milk without any risk. This would allow subsequent adaptation of the milk feeding strategy. Also, because the HCMV contamination peak appears 4 to 8 weeks after birth, the work we present here should lead to a device meant to be used both at hospital and at home in a self-testing manner.

1 INTRODUCTION

Human cytomegalovirus (HCMV), rarely dangerous for immune-competent person, is a real threat for immune-depressed people (organ transplanted or pregnant women). HCMV is the most frequent etiologic agent of congenital and postnatal infection of newborns and can have a significant impact on the neurosensory development of newborns and especially preterm infants (Hayashi et al. 2011). Recent studies showed that postnatal HCMV infection in preterm infants can lead to serious clinical consequences and can lead to death in rare cases (Lanzieri et al. 2013; Hamele et al. 2010; Hamprecht et al. 2008). Although the long-term follow-up of the neurosensory development of congenitally infected preterm infants is well documented, very few studies concern postnatal infected preterm infants. However, actual data suggest a negative influence on long-term cognitive development (Kurath et al. 2010; Bevot et al. 2012; Goelz et al. 2013).

Breastfeeding is now clearly recognized as being superior to artificial feeding. However, breastfeeding plays a major role in the epidemiology of transmission and postnatal HCMV infection. It is now well established that HCMV is excreted in milk from about 80% of seropositive lactating mothers (Kurath et al. 2010). Excretion can start since the first post-partum week and reaches a maximum value 4 to 8 weeks after birth. Mother-to-child transmission generally occurs during this period (Hamprecht et al. 2003, 2008). About 20% of breastfed children are HCMV positive and the contamination risk increases with lactating duration. For preterm infants, the weak transmission of

mother antibodies and the non-mature immune system increases the risk of symptomatic HCMV infection.

Today, almost no national recommendations on the manipulation of the breast milk of HCMV positive mothers are proposed. Methods exist to treat breast milk. Systematic pasteurization is not done because it alters the immune components of milk (Chang et al. 2013). Freezing at -20 °C does not completely destroy the virus (Yoo et al. 2015). Neonatologists are then squeezed in their clinical practice between the potential risk to transmit infection when breast milk is not treated and the risk to favor complications if the milk is treated. The ideal solution would be to differentiate “at risk” and “non at risk” situations in order to treat only the “at risk” cases. This would be extremely more satisfactory than a systematic attitude.

The techniques currently available to detect HCMV are PCR and cell culture but are not adapted to rapid and early HCMV detection in breast milk (Hamprecht et al. 2008). Indeed, PCR is expensive and time consuming and cell culture gives a result only several days after sampling. ELISA can hardly be used because in this static fluidic configuration, nutritive components of the milk shield the antibodies used for detection.

In order to help clinicians in their practice, our project aims at developing a simple, fast and low-cost system namely a rapid diagnostic test (RDT) which could be used at the hospital or at home in a self-evaluation manner.

The biological and technological hypotheses developed in this position paper concern the fact that in a fluid flow configuration, HCMV remains available for bio-recognition by specific antibodies. Using specifically marked detection antibodies, an optical measurement is possible. Prior to the development of a RDT, a technico-clinical study is ongoing to develop an integrated immuno-combined device able to detect HCMV in native breastmilk. The goal is to determine the most suitable antibodies association to be used. In section 2, we show that a fluid flow configuration is much more efficient than ELISA-like methods although the laboratory model we used suffered from instabilities. In section 3, we present the very first results obtained with a stable and reliable integrated device. In part 4, and in line with the scope of a position paper, we present scientific and socio-economic impacts the foreseen RDT (still to be fabricated) could address.

2 PRINCIPLE AND PRELIMINARY EXPERIMENTS

2.1 Biochip principle

The technique we propose to detect HCMV is based on antigen/antibody recognition. Biochips were designed and homemade. The biosensor consists of a polystyrene biochip coated by human polyclonal anti-HCMV antibodies as shown in figure 1. HCMV potentially present in the breast milk sample is captured by these antibodies. HCMV detection uses a specific secondary antibody coupled to horseradish peroxidase (HRP) enzyme which subsequently recognizes the captured virus. After addition of a substrate of this enzyme, a colorimetric reaction occurs and allows transforming the substrate to a blue product. Then, the optical reading relies on an absorbance measurement at about 640 nm or 450 nm if the reaction is stopped with sulfuric acid.

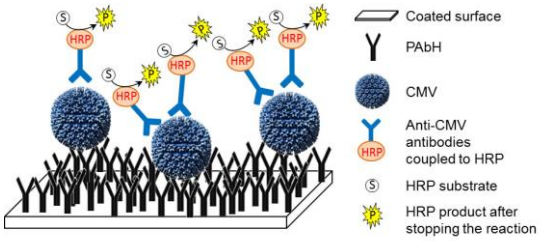

Figure 1: Principle of the HCMV biosensor.

2.2 Preliminary tests

The first HCMV detection tests were conducted with an initial version of a homemade laboratory model in parallel with ELISA (Enzyme-Linked Immunosorbent Assays) experiments. The goal was to compare dynamic (laboratory model) and static (ELISA) conditions in terms of virus detection. The laboratory model consists of a fluidic system containing a biosensor inserted into a cartridge. Syringes contain reagents which are driven on biosensor surface. The system allows controlling the fluid flows and interaction durations. The biosensor is sandwiched between a LED emitting in the red wavelength region and a photodiode as shown in figure 2(a). Initially, the device was designed for 4 simultaneous tests.

Preliminary positive results were obtained with the device from artificially contaminated breast milk samples. Indeed, virus concentrations as low as 6 ng/mL can be detected using this simple opto-fluidic model with a higher absorbance value than the one

obtained with ELISA technique (figure 2(b)). Furthermore, it can be seen that absorbance levels achieved in ELISA are of the same order of magnitude as those obtained using irrelevant antibodies. This highlights the fact that a static configuration is not suitable for virus detection in native breastmilk.

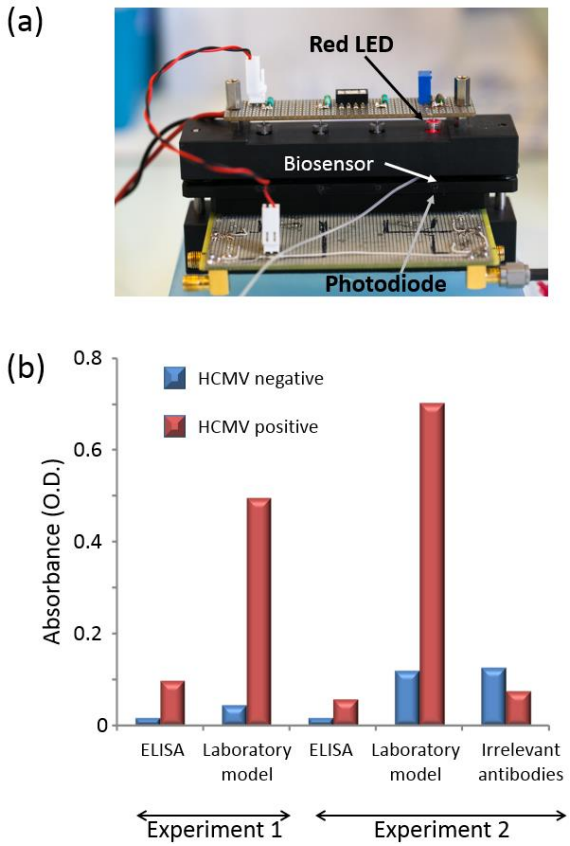

Figure 2: HCMV (~6 ng/mL) detection in artificially contaminated breastmilk in static conditions (ELISA) and dynamic conditions (laboratory model). Two different experiments performed with two distinct batches of capture antibodies are presented.

In view of the first results obtained using breastmilk, dynamic conditions proved to be extremely efficient for HCMV detection. Indeed, optical densities as high as 0.7 are achieved in dynamic conditions against 0.1 maximum in static condition. As previously mentioned, the high proportion of lipids in this biological fluid makes capture and detection of viruses more difficult due to a shield effect of the components. These results should be validated using naturally HCMV contaminated breastmilk sample and with a much larger number of samples.

The laboratory model presented in figure 2 proved to be inappropriate for a large scale study. Indeed, we experienced number of experimental problems. The mechanical stability was not high enough to ensure reproducible measurements. The gap between the LED and the photodiode is open to air and variations in the ambient light jeopardize measurements. Optical densities presented in figure 2(b) were measured after the experiment was finished by means of an optical spectrometer. Therefore, no continuous absorption measurements were possible.

In fact, the laboratory model is a copy of the model used to experiment on red cell immuno-capture (Charrière et al. 2012). Biological reactions involved in these experiments lasts for a few minutes only. Optical absorption of red cells is orders of magnitude higher than the absorption measured in the current experiments. Also, experimentation times were a few minutes for red cells and more than 30 minutes for HCMV. Therefore, variations of the optical power emitted by the LED become an issue. Stabilization of the emitted power is then required in our experiments. This is why we designed a new, compact and opto-mechanically stable prototype which allows continuously recording optical absorption during the experiment.

3 A STABILIZED AND INTEGRATED PROTOTYPE

3.1 Description of the new prototype

A Computer Aided Design of the new prototype is presented in figure 3.

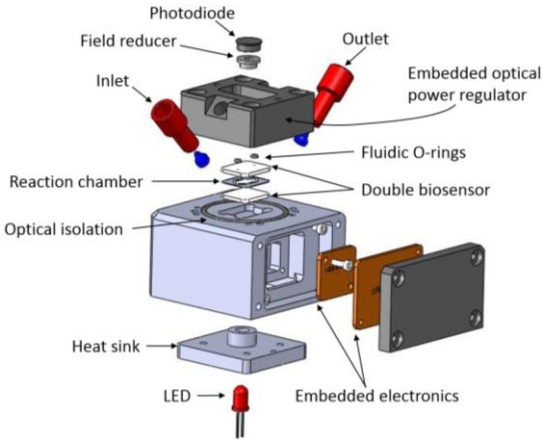

Figure 3: CAD view of the new opto-fluidic prototype.

A reaction chamber defined in a sheet of PDMS is sandwiched between two biosensors which are used to enhance the sensor's sensitivity. Sample and reagents are introduced in and evacuated from the reaction chamber through commercially available microfluidic connectors. Fluid flows are controlled using motorized syringes. Fluidic sealing is ensured using two micro O-rings. Embedded spacers are used to ensure that the thickness of the reaction chamber (few tens of µm) remains constant between experiments (new biosensors must be used for each experiment). Also, embedded stops are used to ensure a vertical repositioning between experiments. Stops and spacers are not visible in the figure.

The optical absorption measurement is provided by the couple LED-photodiode. The LED is embedded in a heat sink in order to reduce heating due to long operations (more than 30 min). Indeed, LED heating results in an increase of the emitted power. Although the heat sink cannot completely stabilized the LED temperature, an optically stable operation is ensured with an embedded electronic optical power regulator. The photodiode is inserted into a spacer. A 2 mm diameter hole is drilled in the spacer. It allows collecting only light which has propagated in straight line from the LED. Isolation from the ambient light is obtained using a large diameter O-ring placed in a circular groove.

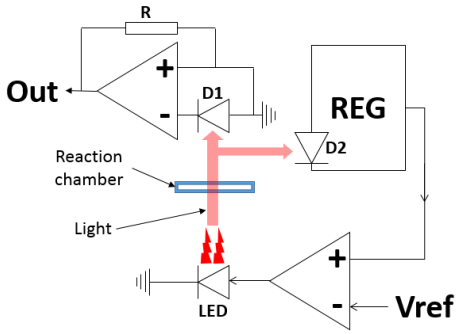

Figure 4: Schematic representation of the driving and detection electronics.

Driving and detection electronics uses surface mount component on PCBs directly integrated in the device. Internal wiring is not shown in the figure. The optical power regulator is integrated in the top part of the prototype while PCBs are included in the bottom part. The general electronic circuit is schematically described in figure 4. D1 is the photodiode which produces the absorption signal while D2 is the photodiode of the regulation circuit. Figure 5 shows the actual prototype with a closer view to the reaction chamber. Stops and light shield are also visible.

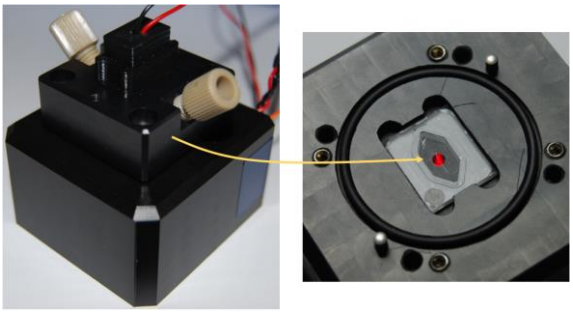

Figure 5: Pictures of the prototype.

3.2 Sample preparation

For this set of experiments, breastmilk samples were not available. Experiments were then conducted using a simplified biological model. Biochips and samples were prepared as follows. Three biochips were coated by human polyclonal anti-HCMV antibodies at a concentration of 20 ng/µL in carbonate/bicarbonate buffer overnight at 4°C. The next day, a rinsing of chips with PBS 1X followed by a saturation step of the surface with Bovine Serum Albumin (BSA) 10% during 1 h at room temperature (RT) was performed. A mixture of commercial CMV antigen (pure or diluted at 1/25) and an anti-CMV antibody conjugated to HRP diluted at 1/5000 (ETI-CYTOK-M reverse plus Diasorin kit) was injected on two of the chips and incubated 1 h at RT. A negative control was realized by incubation of one chip with the antibody alone diluted in PBS. Here, we call CMVneg the negative control solution, CMV1/25 the test solution with the antigen diluted at 1/25 and CMVpure the test solution with no dilution of the antigen. Three washing of 1 mL were realised with a wash solution composed of PBS-Tween (ETI-CYTOK-M reverse plus, Diasorin) and the HRP substrate (hydrogen peroxide and tetramethylbenzidine) was incubated during about 50 min at RT in the prototype. Absorption measurements were collected during this time of incubation.

3.3 Experimental results obtained with the new prototype

Figure 6 shows the photodiode signal recorded over the whole experiment for the CMVneg, CMV1/25 and CMVpure samples.

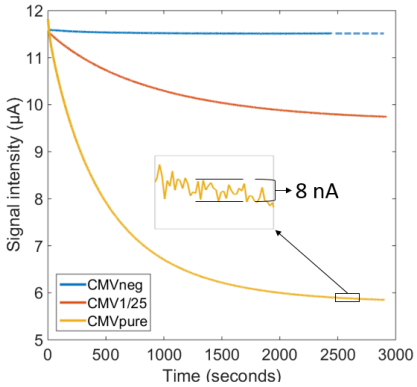

Figure 6: Absorption recorded during the whole experiment session.

It can be observed that the electronic circuits perfectly regulate the LED emitted power. The noise level is extremely small as illustrated in the insert of figure 6. The noise level is of the order of 8 nA. Also, the exponential decay of the signal is clearly observed.

Figure 7 shows the signal recorded at the end of the experiments as a function of the dilution levels: 1/1 CMVpure, 1/25 for CMV1/25 and 1/∞ for CMVneg.

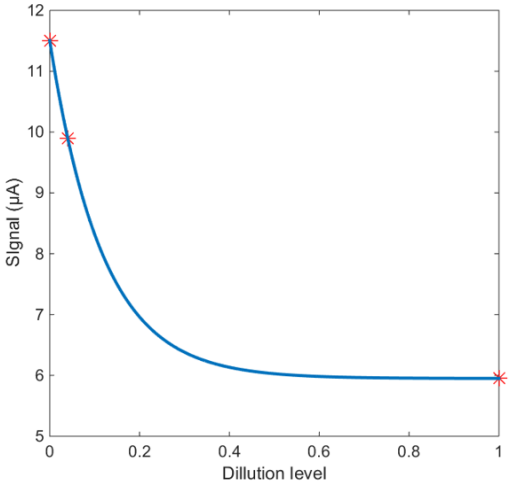

Figure 7: Final signal as a function of the dilution levels.

The blue curve in the figure is an exponential fitting of the data. Of course, this is a very rough approximation because of the very few experimental data. However, it is expected that the viral charge in the actual samples will be quite low. This means that only the left part of the curve should be considered as representative of real situations. Zooming this region of the figure and reporting the noise level allows roughly estimating the detection limit in terms of dilution level (figure 8).

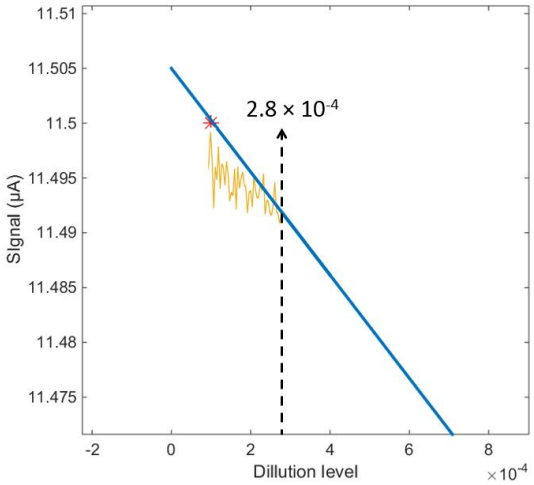

Figure 8: A tentative estimation of the detection limit.

The value of 2.8×10^{-4} represents a sample where the antigen dilution is 1/3500. However, this is nothing but a rough estimation obtained using a simplified biological model. We are now starting a clinical trial in order to assess the accuracy and the potential of the opto-fluidic technique we propose. If successful, a step ahead will have been passed in the direction of a home-use RDT. The impacts of such a device are summarized in the next section.

4 SOCIO-ECONOMICAL IMPACTS

As mentioned above and in the scope of a position paper, we present the scientific and socio-economic impacts such a device potentially.

HCMV is an opportunistic virus that infects a large proportion of the population worldwide and results in an asymptomatic latent infection in healthy subjects. The disease burden is both medical and economic. HCMV infection can lead to severe diseases in the absence of an effective immune response. HCMV is also the leading cause of neonatal viral infection and can have a significant impact on the neurosensory development of newborns and especially preterm infants. HCMV infection may result from maternal-fetal transmission during pregnancy (2-5% of very premature infants) or postnatal transmission (about 20% of children). Currently, viral status of breast milk is not explored in practice and, depending on the health centers, milk is systematically inactivated or breastfeeding is continued with raw milk without any caution. Finally, although the cost of HCMV

infection in the hospital community has not yet been clearly established, it appears that HCMV infections cost hundreds of thousands of euros each year to the French health system in terms of medical and surgical expenses, especially in taking care of long-termed disabled children and adults infected early in life or during pregnancy.

Therefore, an easy-to-use secured RDT to detect HCMV infection in breastmilk from lactating women of preterm infants is urgently needed. An answer to this need is the subject of this communication. About 8000 very preterm infants could benefit from this test each year in France and 13 million worldwide. Considering that the test should be repeated several times for a same couple mother/baby pair in the early months of breastfeeding, the market worth to be taken into account. Indeed, the test will be practiced both at hospital and at home since the peak of viral excretion in breast milk occurs generally after hospitalization of the child. In addition to detection by caregivers in departments of neonatal medicine, self-diagnosis of mothers will be possible given ease of use and reading of this type of test.

5 CONCLUSION

Although the risk of HCMV congenital infection is relatively low, the risk of postnatal contamination, in particular *via* breast milk, can be dramatic for preterm infants. Currently, the question is: should we favor a better development and take the risk of using contaminated breast milk, or should we use treated milk, even when the HCMV infection is low enough to be considered safe?

To address this problem, and in the current context of breastfeeding promotion, we propose to develop a HCMV biosensor based on sandwich ELISA principle in a dynamic flow configuration. This position paper presents studies that have just started, but we think it is possible to set-up an easy to use and rapid "point-of-care" device to detect HCMV in breastmilk. Therefore, a third answer can be proposed to the above mentioned question. The idea is to screen HCMV on a routine basis and to define a personalized feeding strategy for “at risk” population only. Without such a rapid HCMV test, this third solution may never exist.

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REFERENCES

Bevot, A, et al., 2012. Long-Term Outcome in Preterm Children with Human Cytomegalovirus Infection Transmitted via Breast Milk. *Acta Paediatrica* 101 (4): e167-172.

Chang, J.C., et al., 2013. Influence of Prolonged Storage Process, Pasteurization, and Heat Treatment on Biologically-Active Human Milk Proteins. *Pediatrics and Neonatology* 54 (6): 360‑66.

Charrière, K., et al., 2012. SmarTTransfuser: A biochip system for the final ABO compatibility test. *BIODEVICES 2012 - Proceedings of the International Conference on Biomedical Electronics and Devices*, janvier, 257‑62. SCITEPRESS.

Goelz, R., et al., 2013. Long-Term Cognitive and Neurological Outcome of Preterm Infants with Postnatally Acquired CMV Infection through Breast Milk. *Archives of Disease in Childhood. Fetal and Neonatal Edition* 98 (5): F430-433.

Hamele, M., et al., 2010. Severe Morbidity and Mortality with Breast Milk Associated Cytomegalovirus Infection. *The Pediatric Infectious Disease Journal* 29 (1): 84‑86.

Hamprecht, K., et al., 2008. *Cytomegalovirus transmission to preterm infants during lactation*. Journal of Clinical Virology 41 (3): 198‑205.

Hamprecht, K., et al., 2003. Rapid detection and quantification of cell free cytomegalovirus by a high-speed centrifugation-based microculture assay: comparison to longitudinally analyzed viral DNA load and pp67 late transcript during lactation. *Journal of Clinical Virology* 28 (3): 303‑16.

Hayashi, S., et al., 2011. Transmission of cytomegalovirus via breast milk in extremely premature infants. *J Perinatol* 31 (6): 440‑45.

Kurath, S., et al., 2010. Transmission of Cytomegalovirus via Breast Milk to the Prematurely Born Infant: A Systematic Review. *Clinical Microbiology and Infection* 16 (8): 1172‑78.

Lanzieri, T., et al., 2013. Breast Milk-Acquired Cytomegalovirus Infection and Disease in VLBW and Premature Infants. *Pediatrics* 131 (6): e1937-1945.

Yoo, H.S., et al., 2015. Prevention of Cytomegalovirus Transmission via Breast Milk in Extremely Low Birth Weight Infants. Yonsei Medical Journal 56 (4): 998‑1006.