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SHORT COMMUNICATION

Evaluation of CMV DNA in dried blood spot

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SUMMARY

Cytomegalovirus is the primary viral cause of congenital infection. However, diagnosis may be difficult for clinical and technical reasons. Currently, evaluation of CMV DNA on dried blood spot (DBS) is an important instrument to define a congenital infection. The aim of this study was to identify a clinically and technically suitable diagnostic work-flow for CMV DNA evaluation on DBS. Sensitivity was not significantly influenced by storage time of up to 12 months and extraction technique; however, analysis in triplicate was crucial to obtain reliable results. Considering viral load in an infected foetus at risk of developing disease, a threshold value of approximately 10^4 copies/mL was characterized by high operating characteristics for detection of positivity at 12 months on DBS.

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Cytomegalovirus (CMV) is a ubiquitous virus causing asymptomatic or self-limited infection in healthy children and adults, while in immunocompromised hosts and infected foetuses CMV may be responsible for a high burden of disease (Gerna and Lilleri, 2019; Marsico and Kimberlin, 2017). CMV is the most common cause of congenital infections, with incidence rates ranging from 0.2 to 2% of live-born neonates; approximately 10% of congenitally infected infants have signs and symptoms of disease at birth, with a high risk for subsequent development of neurological sequelae, including sensorineural hearing loss, mental retardation, microcephaly, development delay, seizure disorders, and cerebral palsy (Swanson and Schleiss, 2013; van Zuylen *et al*., 2014). CMV is transmitted from mother to foetus in approximately 35% of pregnancies in which a maternal primary infection occurs, with transplacental transmission rates ranging from 20% to 75%, related to the trimester of infection (Bonalumi *et al*., 2011).

Congenital CMV infection may be undetected for different reasons: in pregnant women, CMV infection is often asymptomatic; most infected new-borns are asymptomatic and screening of pregnant women is not recommended by national public health bodies in any country (Gantt *et al.*, 2017; Rawlinson *et al*.,2017; Saldan *et al*., 2016).

Currently, diagnosis of congenital CMV infection is based on the detection in urine, saliva, or blood of CMV DNA via nucleic acid testing (NAT) within the first 2–3 weeks of life (Cannon *et al*., 2014). During the first 2 weeks after birth, blood is routinely collected from the heel or fingertips and is stored as Dried Blood Spots (DBSs) on Guthrie cards. The detection of CMV DNA on stored DBS could be an opportunity to diagnose congenital CMV infection, including at subsequent time periods upon the occurrence of suggestive signs and/or symptoms (Soetens *et al*., 2008). Indeed, different studies evaluated the potential of PCR assay on DBS for screening and/or diagnosing of congenital CMV (Boppana *et al*, 2010; Koontz *et al*., 2015; Meyer *et al*., 2017; Soetens *et al*., 2008; Wang *et al*., 2015). However, DBS PCR tests seem more suitable than screening for retrospective diagnosis of congenital CMV infection (Wang *et al*., 2015). The aim of this study was to identify a clinically and technically suitable diagnostic work-flow for CMV DNA evaluation on Guthrie Card.

To this aim, Guthrie cards prepared by spotting CMV DNA positive whole blood specimens obtained from solid organ transplant patients referred for virological analyses to the Microbiology and Virology Unit, University Hospital "Città della Salute e della Scienza di Torino," were studied. In particular, ten specimens for each order of magnitude of CMV viral load ranging from 10^2 to 10^6 copies/mL, accounting for a total of 50 Guthrie cards, were prepared by dispensing 50 µL (about the volume of a drop) of blood onto the paper. Spots were then dried for at least 24 hours at room temperature and stored at the same temperature for two different durations: 1 month and 12 months.

Nucleic acid extraction was performed by the automatic QIAsymphony platform (Qiagen, Germany) also comparing two different methods: DNA Investigator kit Qiasymphony® (Reference 200V5 protocol) (investigator method) and DSP Virus/Pathogen midi-kit Qiasymphony® (Pathogen Complex 400V4 protocol) (standard method). Both methods were evaluated in single and triplicate in order to assess sensitivity for the different viral loads and the two storage periods. Sample input for each method was 3 punches of 3 mm in size (area of 7.07 mm^2).

For CMV DNA amplification, a commercially-available real time PCR assay targeting a region of the exon 4 of major immediate early antigen (MIEA) gene (CMV ELITe MGB® Kit, ElitechGroup, Italy) was used; amplification was performed on a 7500 Real-Time thermo-cycler system (Applied Biosystems, USA).

All amplifications were performed in single and in tripli-

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Table 1 - *Sensitivity of CMV DNA detection on dried blood spot according to initial viral load and time of storage.*

		Month 1		Month 12	
		Investigator Method	*Standard Method*	*Investigator Method*	*Standard Method*
10^2	*Single*	0%	10%	0%	20%
	Triplicate	20%	20%	0%	30%
10^3	*Single*	20%	40%	10%	30%
	Triplicate	50%	50%	30%	40%
10^4	*Single*	30%	70%	80%	90%
	Triplicate	40%	90%	80%	100%
10^5	*Single*	100%	100%	90%	100%
	Triplicate	100%	100%	100%	100%
10^6	*Single*	100%	100%	100%	100%
	Triplicate	100%	100%	100%	100%

cate; the sample was considered positive when at least one of the three triplicate amplifications was positive.
By evaluating increasing initial viral load, data showed 100% sensitivity, at either single or triplicate analyses, for both extraction methods from 10^6 to 10^5 copies/mL at 1 month, and only for 10^6 copies/ml at 12 months. Occurrence of negative results at single evaluation was observed at 10^2 copies/mL with the investigator method at both 1 month and 12 months. Negative results recurred both in single and triplicate analysis at 12 months with the investigator method. Overall, sensitivity tended to reduce with decreasing viral loads for both methods, but showed higher rates for standard method. Sensitivity rates are summarized in *Table 1*. Evaluation of an intersection point with high specificity and sensitivity to identify CMV DNA threshold for detection of positivity at 12 months was made by ROC curve analysis and allowed to define 9098 copies/mL as the viral load above which the positivity is likely to be detected on DBS (*Figure 1*).

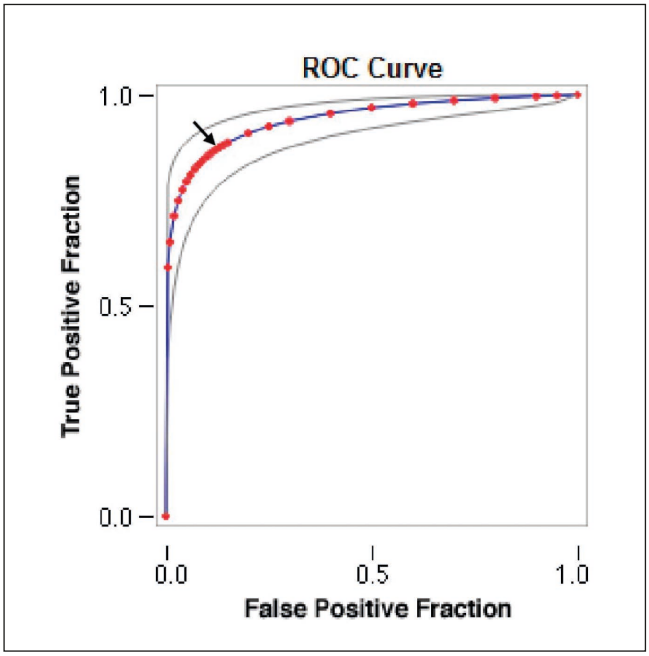

Figure 1 - *Identification of a CMV DNA viral load (copies/mL) threshold for positivity at 12 months from DBS collection by ROC curve analysis. A threshold of 9098 copies/mL of initial viral load on Guthrie card corresponds to 0.892 sensitivity (y axis) and 0.877 specificity (x axis); area under the curve 0.943.*

Based on comparison of the two extraction methods, no significant difference in terms of sensitivity was observed, but only a tendency to higher results for the standard method. Therefore, both methods can be used without loss of performance, thus taking into consideration the most suitable method in relation to routine laboratory asset. However, we noticed relevant inconsistencies in terms of sensitivity rate for viral loads lower than or equal to 10^4 copies/mL, but not for higher viral loads. Different studies have compared the sensitivity of manual and automated extraction methods (Göhring *et al.*, 2010; Koontz *et al.*, 2015; Koontz *et al.*, 2019; Vauloup-Fellous *et al.*, 2007) and the results showed that the best performance was obtained with manual methods. However, this should not be suitable for clinical settings that need automation. Interestingly, the study of Koontz *et al.* (2015) showed that the investigator kit was significantly better in terms of extraction compared to the DSP Virus/Pathogen midi-kit.
Also, the ROC curve derived from data showed suitable operational characteristics only for viral loads higher than or equal to 10^4 copies/mL. It is known that, in general, CMV intrauterine infection should cause post-birth sequelae with high viral loads in blood, whereas low viral loads are unlikely to be associated with clinical consequences for children (Enders *et al.*, 2017; Forner *et al.*, 2014). Although there isn't always a correlation between viral load and clinical sequelae, the risk of sensorineural hearing loss increased with DBS viral load (Walter *et al.*, 2008). For these reasons, we assumed that the limit of sensitivity of 9098 copies/mL can be considered acceptable for the clinical use.
Although no difference in terms of sensitivity emerged between the two methods, analysis in triplicate is crucial to achieve better results. In this way, according to other studies (de Vries *et al., 2009;* Koontz *et al.,* 2015), sensitivity should be increased and there are fewer risks of obtaining false negative results, as instead may happen with single, especially at the lowest viral loads which are around the detection limits of automated extraction methods. It was also observed that storage time does not significantly affect analytical sensitivity, and this feature should be taken into account in consideration of the fact that diagnosis of congenital CMV infection can be requested a long time after the sample collection on DBS.
This work was intended as a preliminary study, in order to evaluate if the extraction method routinely used in our laboratory could be applied to DBS analysis without loss of sensitivity and necessity to use different extraction kits. The most important limit of this work is the number of samples tested. With a higher number of data, it will be possible to conduct a significant evaluation of differences between the two methods. Another consideration that deserves further study is the relationship between whole blood and DBS samples, especially for evaluation of the dilution factor between the two sample matrixes.

References

Bonalumi S., Trapanese A., Santamaria A., D'Emidio L., Mobili L. (2011). Cytomegalovirus infection in pregnancy: review of the literature. *J Prenat Med.* **5**, 1-8.
Boppana S.B., Ross S.A., Novak Z., et al. (2010). Dried blood spot real-time polymerase chain reaction assays to screen newborns for congenital cytomegalovirus infection. *JAMA*. **303**, 1375-1382.

Cannon M.J., Griffiths P.D., Aston V., Rawlinson W.D. (2014). Universal newborn screening for congenital CMV infection: what is the evidence of potential benefit? *Rev Med Virol.* **24,** 291-307.

de Vries J.J.C., Claas E.C.J., Kroes A.C.M., Vossen Ann C.T.M. (2009). Evaluation of DNA extraction methods for dried blood spots in the diagnosis of congenital cytomegalovirus infection. *J Clin Virol.* **46**, S37-S42.

Enders M., Daiminger A., Exler S., Ertan K., Enders G., Bald R. (2017). Prenatal diagnosis of congenital cytomegalovirus infection in 115 cases: a 5 years' single center experience. *Prenat Diagn.* **37**, 389-398.

Forner G., Abate D., Mengoli C., Palù G., Gussetti N. (2014). High Cytomegalovirus (CMV) DNAemia Predicts CMV Sequelae in Asymptomatic Congenitally Infected Newborns Born to Women with Primary Infection During Pregnancy. *J Infect Dis.* **212**, 67-71.

Gantt S., Bitnun A., Renaud C., Kakkar F., Vaudry W. (2017). Diagnosis and management of infants with congenital cytomegalovirus infection. *Paediatr Child Health.* **22**, 72-74.

Gerna G., Lilleri D. (2019). Human cytomegalovirus (HCMV) infection/re-infection: development of a protective HCMV vaccine. *New Microbiol.* **42**, 1-20.

Göhring K., Dietz K., Hartleif S., Jahn G., Hamprecht K. (2010). Influence of different extraction methods and PCR techniques on the sensitivity of HCMV-DNA detection in dried blood spot (DBS) filter cards. *J Clin Virol.* **48**, 278-281.

Koontz D., Baecher K., Amin M., Nikolova S., Gallagher M., Dollard S. (2015). Evaluation of DNA extraction methods for the detection of Cytomegalovirus in dried blood spots. *J Clin Virol.* **66**, 95-99.

Koontz D., Dollard S., Cordovado S. (2019). Evaluation of rapid and sensitive DNA extraction methods for detection of cytomegalovirus in dried blood spots. *J Virol Methods.* **265**, 117-120.

Marsico C., Kimberlin D.W. (2017). Congenital Cytomegalovirus infection: advances and challenges in diagnosis, prevention and treatment. *Ital J Pediatr.* **43**, 38.

Meyer L., Sharon B., Huang T.C., Meyer A.C., Gravel K.E., Schimmenti L.A., et al. (2017). Analysis of archived newborn dried blood spots (DBS) identifies congenital cytomegalovirus as a major cause of unexplained pediatric sensorineural hearing loss. *Am J Otolaryngol*. **38**, 565-570

Rawlinson W.D., Boppana S.B., Fowler K.B, Kimberlin D.W., Lazzarotto T., Alain S., et al. (2017). Congenital cytomegalovirus infection in pregnancy and the neonate: consensus recommendations for prevention, diagnosis, and therapy. *Lancet Infect Dis*. **17**, e177-e188.

Saldan A., Forner G., Mengoli C., Gussetti N., Palù G., Abate D. (2016). Testing for Cytomegalovirus in Pregnancy. *J Clin Microbiol*. **55**, 693-702.

Soetens O., Vauloup-Fellous C., Foulon I., Dubreuil P., De Saeger B., Grangeot-Keros L. Naessens A. (2008). Evaluation of Different Cytomegalovirus (CMV) DNA PCR Protocols for Analysis of Dried Blood Spots from Consecutive Cases of Neonates with Congenital CMV Infections. *J Clin Microbiol*. **46**, 943-946.

Swanson E.C., Schleiss M.R. (2013). Congenital Cytomegalovirus Infection. *Pediatr Clin North Am*. **60**, 335-349.

van Zuylen W.J., Hamilton S.T., Naing Z., Hall B., Shand A., Rawlinson W. (2014). Congenital cytomegalovirus infection: Clinical presentation, epidemiology, diagnosis and prevention. *Obstet Med*. **7**, 140-146.

Vauloup-Fellous C., Ducroux A., Couloigner V., Marlin S., Picone O., Galimand J., Loundon N., Denoyelle F., Grangeot-Keros L., Leruez-Ville M. (2007). Evaluation of cytomegalovirus (CMV) DNA quantification in dried blood spots: retrospective study of CMV congenital infection. *J Clin Microbiol*. **45**, 3804-3806.

Walter S., Atkinson C., Sharland M., Rice P., Raglan E., Emery V.C., Griffiths P.D. (2008). Congenital cytomegalovirus: association between dried blood spot viral load and hearing loss. *Arch Dis Child Fetal Neonatal Ed*. **93**, F280-F285.

Wang L., Xu X., Zhang H., Qian J., Zhu J. (2015). Dried blood spots PCR assays to screen congenital cytomegalovirus infection: a meta-analysis. *Virol J*. **12**, 60.