American Scientific Research Journal for Engineering, Technology, and Sciences (ASRJETS)
ISSN (Print) 2313-4410, ISSN (Online) 2313-4402

http://asrjetsjournal.org/

Dried Blood Spots as a Clinical Samples for Laboratory Diagnosis and Surveillance of Vaccine-Preventable Diseases in Bulgaria

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Abstract

In recent years the dried blood spots (DBS) had new and innovative applications in medicine, neonatology, virology and microbiology. This study aimed to evaluation of the frequency of detection of viral IgM/IgG markers in dried blood spots and introducing an easy-to-implement protocol for serum extraction in measles, mumps and rubella surveillance. The total 204 clinical samples (102 serum samples and 102 dried blood spots) collected from 102 patients were included. All specimens were tested for presence of specific viral markers (IgM and IgG antibodies) by a commercial indirect enzyme-linked immunosorbent assay (ELISA). Of all tested patients, three (3/102, 2.94%, 95% CI: 0 ÷ 6.22) were confirmed for acute measles infection and two (2/102, 1.96%, 95% CI: 0 ÷ 4.65) for mumps. Double positive ELISA-IgM results were found in their serum samples and DBS. No acute rubella infection and rubella IgM marker were detected in both clinical samples. By immunoassay analysis of all 102 patients, measles, mumps and rubella IgG were found in 83/102 (81%, 95% CI: 73.40 ÷ 88.60), 76/102 (75%, 95% CI: 66.60 ÷ 83.40) and 79/102 (77%, 95% CI: 68.83 ÷ 85.17) serum samples. Comparative results were obtained in the adequately obtained DBS. Viral IgG seroprevalence in DBS were obtained in 79/102 (77%, 95% CI: 68.83 ÷ 85.17) for measles, 69/102 (68%, 95% CI: 58.67 ÷ 77.33) for mumps and 73/102 (72%, 95% CI: 63 ÷ 81) for rubella, respectively. Double negative results for each screened viral markers were proven in six tested patients.

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The study shown higher extinction value (Ratio and NovaTec units) in DBS compared to serum samples of same persons were calculated. Our studies show over 90% coincidence in combined ELISA assay of viral markers against measles, mumps, and rubella in serum samples and DBS. DBS clinical approach is non-aggressive and more acceptable to the public (including young children, pregnant women, etc.). It has a variety of new and innovative applications in medicine and in particular in the laboratory diagnosis of acute and past (presence of protective immunity) measles, mumps and rubella infection in the phase of elimination.

Key words: dried blood spots; vaccine-preventable diseases; measles; mumps; rubella; ELISA IgM/IgG.

1. Introduction

Traditionally, laboratory diagnosis of vaccine-preventable diseases measles, mumps and rubella is based on the detection of IgM and IgG antibodies by enzyme-linked immunosorbent assay (ELISA) in blood samples obtained by routine venous blood collection. However, the use of capillary whole blood in dried blood spot (DBS) specimens may be an excellent alternative in serological tests and a range of epidemiological studies for the screening of these infections, since these approaches are less invasive, samples can be stored and transported without refrigeration, and highly trained personnel are not necessary. The use of DBS may be considered where a reliable cold chain is not available or logistical barriers for efficient transport of serum exist because IgM and IgG antibodies are stable once the blood has dried on filter paper [1]. The added advantage is that DBS can also be utilized to detect both measles and rubella nucleic acid by Polymerase Chain Reaction (PCR) assays when collected soon after disease onset [1, 2]. The use and scientific data about dried blood spots in virology are scarce, mainly due to the limitations of sensitivity and specificity in the screening of such small volumes of blood (equivalent to 5-10 U / mL) [3-5].

DBS assay could be successfully applied not only in the detection of acute infection but also in implementation of complex seroepidemiological studies, for assessing the post vaccine status of a set of vaccine preventative diseases and other significant public health pathogens. The approach is non-aggressive and more acceptable to the public (including young children, newborns, pregnant women, etc.) [6-10].

The process of measles and rubella elimination is often disturbed by timely laboratory diagnosis and screening of difficult-to-reach risk groups. This problem can be avoided by using alternative clinical material, which collection that does not require trained staff, with a minimal risk of bacterial contamination or haemolysis, and with a long storage time without impact on the quality of the analysis. The use and scientific data about DBS in virology are scarce, mainly due to the limitations of sensitivity and specificity in the screening of such small volumes of blood and the need to optimize working protocols for detection of different markers [6-10].

This study **aimed** to evaluation of the frequency of detection of viral IgM/IgG markers in dried blood spots and introducing an easy-to-implement protocol for serum extraction in vaccine-preventable diseases surveillance.

2. Materials and Methods

2.1. Patients and specimens

The total 102 patients with two types of clinical material (102 serum samples and 102 dried blood spots) were tested. The specimens were collected according to a research project funded by the National Science Fund, Bulgaria, Contract №DM 03/1,12.12.2016. All of them were laboratory tested at the National Reference Laboratory (NRL) "Measles, Mumps and Rubella" of the National Center of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria.

From all patients were collected two types of clinical materials: blood specimens by venipuncture and dried blood spots by pricking a finger or heel using sterile automatic lancets (1.5 - 2 mm). Blood was centrifuged at 4000 rpm for 10 minutes and serum was aliquoted and frozen at -20°C until analyzed. Blood stains were stored on the cards of filter paper (HiMedia`s InstaDNA TM Cards), labeled and drying at room temperature for 30 minutes and storage in ziplock bags with desiccant to 2^0C - 8^0C.

Patient age was recorded from 102 patients and the mean ± SD age was 36.48±24.49 years, and median age was 39 years (range 2 months – 81 years). The majority of patients were from capital Sofia and two other major Bulgarian cities Plovdiv and Burgas.

2.2. Serological analysis

All specimens were tested for presence of specific viral markers by a commercial indirect enzyme-linked immunosorbent assay. Samples were screened for: anti-Measles IgM/IgG (Anti-Measles IgM/IgG ELISA, Euroimmun, Germany), anti-Rubella IgM/IgG (Anti-Rubella, IgG/IgM, Euroimmun, Germany) and anti-Mumps IgM/IgG (NovaLisa Mumps Virus IgM/IgG, ELISA Kit, NovaTec Immundiagnostica, Germany) antibodies. The tests were carried out according to manufacturer's instructions. The absorbance values of tested samples were compared by the mean absorbance values of calibrators and the results were interpreted qualitatively as positive, negative or equivocal for measles, mumps and rubella. According to the instructions of the diagnostic kits, for the critical calculation values of measles and rubella IgM/IgG was adopted R (ratio) = 0.8 and for mumps IgM/IgG - 10 NTU/ml.

2.3. DBS-based anti-Measles, Mumps and Rubella IgM/IgG elution and serological assays

Two 6-mm discs were punched from the DBS cards and added to 200 µl of solution with 0.05% Tween in PBS at room temperature and were placed on a shaker for 1 hour. Samples were incubated overnight at 4°C. The next day, they were centrifuged at 2000 rpm for 10 minutes and incubated 15 minutes at room temperature. The filter paper was removed and DBS eluates were tested. The dilution of eluates that we practiced as optimal was 1:9 or 10 µl DBS eluate and 90 µl Sample buffer (from the test kit).

2.4. Statistical Analysis

For the statistical processing of the results obtained we used relative percentages (%), confidence interval (95% CI), graphical and table analysis.

3. Results

During the period January 2017 - October 2018, a total 102 patient were screened for the presence of specific viral markers, which are indicators for acute (IgM antibodies) or past (IgG antibodies) infections. Two type clinical materials was collected and tested from each patient. The tested person were divided into 11 age groups and they were from three regional cities in Bulgaria – Sofia capital, Plovdiv and Burgas. 51/102 (50%, 95% CI: 59.7 ÷ 40.3) of tested were in age groups 1-4 and ≥50 years old and with data about previous infection or vaccination against measles, mumps, rubella. More males (n=54, 52.94%, 95% CI: 49.91 ÷ 57.97) than females (n=48, 47.06%, 95% CI: 37.36 ÷ 56.76) were tested (Table 1).

Table 1: Distribution of the patients studied by age groups and gender (n=102)

Age groups	Number tested	Male	Female
1-4	20	13	7
5-9	10	9	1
10-14	1	1	0
15-19	0	0	0
20-24	3	2	1
25-29	8	1	7
30-34	5	3	2
35-39	10	2	8
40-44	7	5	2
45-49	7	4	3
≥50	31	14	17
Total	**102**	**54**	**48**

Of all tested patients, three (3/102, 2.94%, 95% CI: 0 ÷ 6.22) were confirmed for acute measles infection and two (2/102, 1.96%, 95% CI: 0 ÷ 4.65) for mumps. Double positive ELISA-IgM results were found in their serum samples and DBS. No acute rubella infection and rubella IgM marker were detected in both clinical samples.

These results corresponded to the clinical manifestation of the tested people, 12 of all 102 were diagnosed with a possible measles infection, 5 with Parotitis epidemica and none with rubella infection.

By immunoassay analysis of all 102 patients, measles, mumps and rubella IgG were found in 83/102 (81%, 95% CI: 73.40 ÷ 88.60), 76/102 (75%, 95% CI: 66.60 ÷ 83.40) and 79/102 (77%, 95% CI: 68.83 ÷ 85.17) serum samples. Comparative results were obtained in the adequately obtained DBS.

Viral IgG seroprevalence in DBS were obtained in 79/102 (77%, 95% CI: 68.83 ÷ 85.17) for measles, 69/102 (68%, 95% CI: 58.67 ÷ 77.33) for mumps and 73/102 (72%, 95% CI: 63 ÷ 81) for rubella, respectively (Table 2).

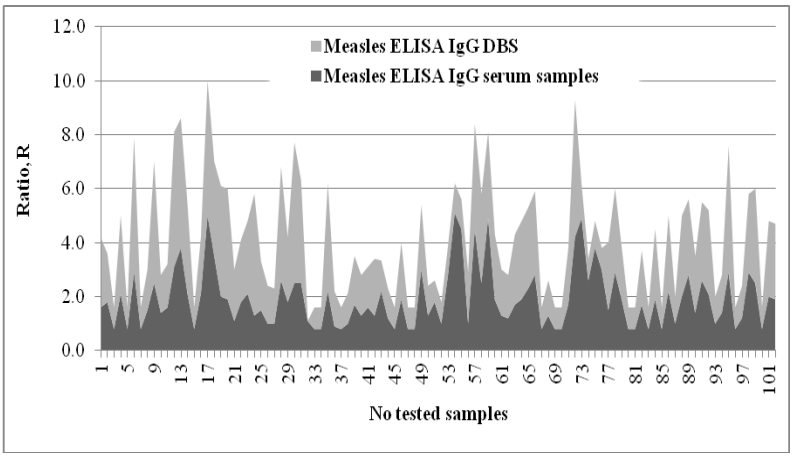
Table 2: Summary of measles, mumps, rubella virus-specific IgM and IgG results for tested serum/DBS sample pairs by ELISA (n=102)

Antibody (interpretation)		**Measles**		**Mumps**		**Rubella**	
		Results for serum samples (No, %)	**Results for DBS (No, %)**	**Results for serum samples (No, %)**	**Results for DBS (No, %)**	**Results for serum samples (No, %)**	**Results for DBS (No, %)**
IgM	Positive	3/102 (**2.94%**)	3/102 (**2.94%**)	2/102 (**1.96%**)	2/102 (**1.96%**)	0/102 (**0%**)	0/102 (**0%**)
	Negative	99/102 (**97.06%**)	99/102 (**97.06%**)	99/102 (**97.06%**)	98/102 (**96.08%**)	100/102 (**98.04%**)	102/102 (**100%**)
	Indeterminate	0/102 (**0%**)	0/102 (**0%**)	1/102 (**0.98%**)	2/102 (**1.96%**)	2/102 (**1.96%**)	0/102 (**0%**)
IgG	Positive	83/102 (**81.37%**)	79/102 (**77.45%**)	76/102 (**74.51%**)	69/102 (**67.65%**)	79/102 (**77.45%**)	73/102 (**71.57%**)
	Negative	11/102 (**10.78%**)	20/102 (**19.61%**)	22/102 (**21.57%**)	23/102 (**22.55%**)	23/102 (**22.55%**)	26/102 (**25.49%**)
	Indeterminate	8/102 (**7.84%**)	3/102 (**2.94%**)	4/102 (**3.92%**)	10/102 (**9.80%**)	0/102 (**0%**)	3/102 (**2.94%**)

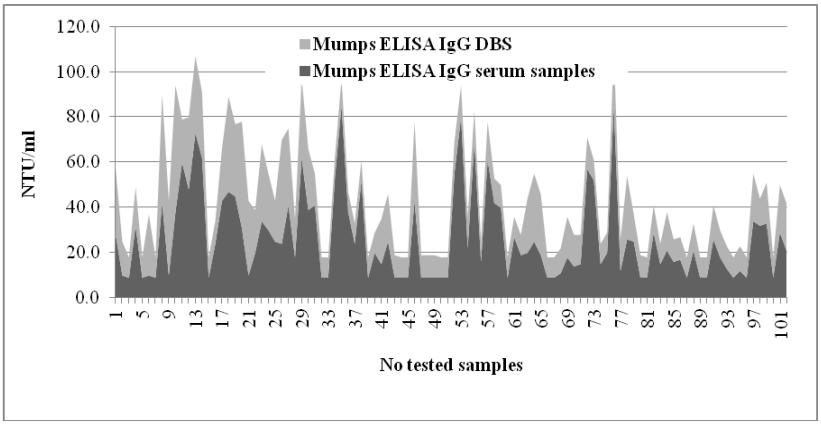
Double negative results for each screened viral markers were proven in six tested patients. These were children under the age of vaccination and no viral infected.

In the course of the study, we compared the calculated Ratio units for anti-measles and anti-rubella IgG and NovaTec units (NTU/ml) for anti-mumps IgG in using serum samples and DBS as starting specimen.

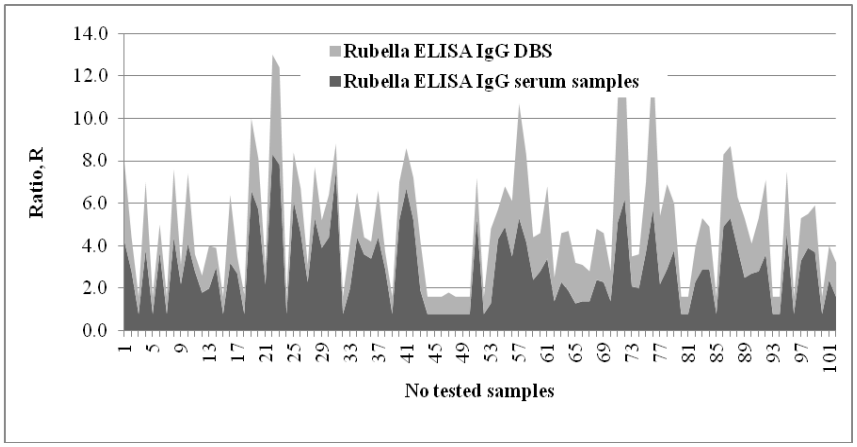
We calculated higher values of Ratio and NovaTec units in used DBS compared to their serum samples (Figure 1). This can be explained by fall of blood forming elements into elution liquid when serum was eluted from the blood spot and possibly enhancing of extinction value.



(A)



(B)



(C)

Figure 1: Comparison of the measles, mumps and rubella virus-specific IgG for serum/DBS sample pairs. Calculated: A) measles (Ratio, R), B) mumps (NTU/ml), and C) rubella (Ratio, R) IgG antibodies in serum samples and DBS by ELISA

Our studies show over 90% coincidence in combined immunoenzymatic testing of viral markers for acute and past measles, mumps, and rubella infection in serum samples and DBS.

4. Discussion

In the past few years, DBS analysis makes procession in clinical laboratory diagnostics and chemistry. In some countries has formulated a national standard for the collection of blood on filter paper as newborn screening including for congenital metabolic diseases [10]. While routine blood collection (venipuncture) can be used to prepare DBS, in general, DBS samples are prepared using capillary blood, a less invasive blood collection method that may be perceived as more acceptable, particularly for young children and infants. In recent years is increasing the application of DBS as method for seroepidemiological survey on viral infections which are subject to enhanced surveillance and with health and socio-economic importance. Namely, vaccine preventable diseases like measles, mumps and rubella. This study is aimed precisely at these areas of application of DBS in laboratory practice. It involves testing a total of 204 clinical samples collected from 102 patients. By indirect ELISA assay, six viral markers have been proven in serum samples and DBS. Despite the small number of positive acute infection markers (3 for measles, 2 for mumps and zero for rubella), 100% coincidence of the results obtained is shown using two types of clinical material. And for the three tested viruses, with a greater percentage (relative sensitivity) are proven IgG in serum samples compared with the DBS of the same persons. This may be explained by an inability to capture or elution of some of the immunoglobulins on filter membranes (recommended for this type of research is Whatman 903) which used and requires future optimization of protocols. Serum IgG seroprevalence was reported in the highest percentage for measles – 81% and lowest for mumps – 76%. The highest percentage (96%) of identical results was obtained by demonstrating measles IgG antibodies in serum samples and DBS. The optical densities (ODs) of 102 DBS samples were compared to the ODs of the corresponding serum samples collected at the same time. The samples were stored for up to 24 months, DBS at 4 - 8°C and serum at -20^{0}C and were tested by commercial IgM/IgG immunoassays. Elution and testing conditions were optimized with the use of DBS. The assay showed an overall sensitivity and a specificity more of 90% for DBS samples compared to the results for serum. The results were analyzed according to the length of time that the DBS sample had been stored and the assay was 98% identical for those samples stored for 6 and 24 months compared to the results for the corresponding serum samples. Similar to our research Helfand, R.F and his colleagues [11] and Karapanagiotidis T., et. al., [12] reported about successfully application of DBS technique to measles cases, and there is accumulating evidence that the technique will work as well for rubella cases. Uzicanin A. et. al. [13] and Helfand R. et. al. [14] published studies seroepidemiological importance of DBS in control and monitoring of measles and rubella infection. The authors reported test sensitivity and specificity over 90% and prove their real alternative to serum samples, particularly in epidemic situations. Recently in the WHO Measles and Rubella Laboratory Network the use of dried blood spots start to validated as useful tools as an alternative to serum for the measles/rubella program in a range of epidemiological studies [2, 11, 12 and 15]. Our results confirm the potential use of the DBS technique in the laboratory diagnosis of acute and past (presence of protective immunity) measles, mumps and rubella infection. In combination immunoenzymatic testing of IgM/IgG markers coincidence for both types of clinical materials were found in ≥90%. The study covered a two-year period in which several measles epidemic outbreaks, sporadic mumps and zero rubella cases have been reported in Bulgaria. Patient samples were selectively

collected, mainly a healthy vaccinated people. There are not many literary data available to demonstrate mumps IgM/IgG markers in DBS, so the present study has the potential for future broader investigation. The assays applied in the present study cover only three vaccine-preventable diseases (measles, mumps and rubella) and also included a relatively small number of tested patients (102). Despite the so mentioned limitations of the study, the tested samples panel was carefully selected and analyzed samples during a period of 24 months, results described here showed the potential role of DBS in proving viral markers with standard immunoassay and optimized the serum elution protocol. DBS technique can be used to overcome some of the challenges of measles and rubella laboratory diagnostic in the era of elimination.

5. Recommendations

In the study of certain patient groups (infants and pregnant women), especially when conducting routine immune status tests, DBS from peripheral blood (finger or heel) can be used as a clinical material. The assay is less invasive, do not require a special transport chain and storage, and the laboratory results for detection of IgG antibodies are similar to those obtained using venous blood. The results should be commented on based on the recommendations of the manufacturers of the kits used and the vaccine status of the patients

6. Conclusion

DBS approach is non-aggressive and more acceptable to the public (including young children, pregnant women, etc.), it has a variety of new and innovative applications in medicine.

Acknowledgements

This study was conducted with the assistance of project № DM 03/1, 12.12.16 “Dried blood spots (DBS) as alternative, archive material for detection of viral agents (measles, mumps, rubella, hepatitis B virus) in Bulgarian population”, „COMPETITION FOR FINANCIAL SUPPORT FOR PROJECT OF JUNIOR RESEARCHERS – 2016”, National Science Fund, Bulgaria.

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