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A Review on Dried Blood Spots (DBS) as Alternative, Archival Material for Detection of Viral Agents (Measles, Mumps, Rubella, Hepatitis B Virus)

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Abstract

In recent years there appears a variety of new and innovative applications of the dried blood spots. The areas of their range of application are medicine, neonatology, virology, microbiology, toxicology and pharmacokinetics, metabolic exchange, therapeutic drug monitoring, toxicology, and control of environmental pollution. The advantages of DBS technology can be combined into four main groups: (1) compared to conventional venipuncture, requires less blood volume, which is especially important in pediatrics and neonatology; (2) the procedure for blood collection is easy, inexpensive and noninvasive; (3) the risk of bacterial contamination or hemolysis is minimal; and (4) DBS can be maintained for a long time with almost no impact on the quality of the analysis. In recent years is increasing the application of DBS as method for seroepidemiological survey with focus viral infections: measles, mumps, rubella and hepatitis B virus. The DBS technique is optimized as an alternative approach (non-invasive, inexpensive, not requiring trained staff and cold chain for transport and storage) of venipuncture collection of clinical material in virology.

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This method facilitates the scientific researches about the concentration of virus specific antibodies in peripheral blood taken from a finger or heel; determining the percentage susceptibility / protection of the studied group of patients againt vaccine-preventable infectious - measles, mumps, rubella and hepatitis B; social benefits - non-invasive technique for testing of small children and infants and applications in regions in the countries with not well developed logistics infrastructure.

Keywords: dried blood spots; viral agents; serological assay; molecular test.

1. Introduction

The idea of using blood collected on paper map made of cellulose attributed to Ivar Christian Bang (1869 - 1918), considered the founder of modern clinical microanalysis [1; 2]. In 1913, Bang determine glucose in eluates from dried blood spots (DBS) and performs measurements of nitrogen using the Kjeldahl method again with the technique of filter paper [2; 3]. Subsequently, several researchers reported the use of dry blood spots in serological tests for the diagnosis of syphilis [2]. In 1924, Chapman [4] summarizes the advantages of DBS technique, stressing four key items that are still valid today: (1) compared to conventional venepuncture, requires less blood volume, which is especially important in areas such as paediatrics and neonatology; (2) the procedure for blood collection is easy, inexpensive and non-invasive; (3) the risk of bacterial contamination or haemolysis is minimal; and (4) DBS can be maintained for a long time with almost no impact on the quality of the analysis. Besides their use for detection of syphilis, subsequently began to talk about applications of this technique for the detection of antibodies against measles, mumps, polio virus, parainfluenza virus and respiratory syncytial virus in 1953 [2] and also for the identification of Shigella in stool dried (feces) on filter paper [5]. In 1969/70s Guthrie published his famous method for neonatal screening for phenylketonuria of DBS obtained by pricking the heel of newborns [6] later and surveillance of congenital hypothyroidism and sickle cell disorders [6; 7]. Approach using capillary blood obtained by heel or finger and soaked on filter paper (Guthrie card), applies to screen metabolic disease in a large population of newborns in Scotland and Susie Guthrie in 1963 [6]. Another type of application stored on filter paper blood is the detection of markers of a disease, such as acyl CoA dehydrogenase [8], human chorionic gonadotropin in cases of Down syndrome [9], and glycated haemoglobin in insulin dependent diabetes mellitus [10] as well as for evaluation of drug levels [11; 12; 13].

2. The applications of DBS in medicine and laboratory diagnostics

There are many reports of the applicability of DBS in medicine. Since 2005 again research focus to DBS, as builds a variety of new and innovative applications. This led to an almost exponential increase in the number of scientific publications in the field of DBS. Areas that fall within their range are toxicology and pharmacokinetics, metabolic exchange, therapeutic drug monitoring, forensic toxicology studies, and control of pollution [14; 15]. 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) [12; 16; 17] (Table 1).

Table 1: Type analysis by DBS technique (according to http://www.spotonsciences.com/dbstechnology/)

Class Molecules	Specific Analysis
Small molecules	amino acids, drugs, hormones, peptides
Nucleotides	DNA, miRNA, mRNA, RNA, virus DNA, virus RNA
Proteins	hemoglobin, cytokines, immunoglobulins, etc.

In the past few years, DBS analysis makes triumphant 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 [18] but the preanalytic phase of DBS analysis is undervalued and not well optimized. Dried blood spot sampling has been used to screen newborn babies for congenital metabolic diseases for over 50 years. A spot of blood from a heel stick of the infant is placed on filter paper and allowed to air dry.

A circular punch (about 3 mm) is removed, eluted with solvent and analyzed for metabolic markers. More than 50 separate analyses can be measured from a quarter size spot of blood, mainly due to adoption within the last 5 years of mass spectrometry for analysis.

DBS sampling also contributes to the generation of higher quality data in pre-clinical studies because more time points can be added without the need for additional rodents and the technology allows for serial pharmacokinetic profiling. In addition, DBS allows for pre-clinical juvenile toxicology studies to be conducted in small animals where the availability of blood has always been a problem.

Advantages of DBS sampling include minimal volume requirements (approximately 30 – 100 µL per spot), ease of sample attainment by finger or heel stick with minimal training required, and ease of transport and sample stability. Once dried, the sample is stable for months to years at ambient temperature or under refrigeration [19; 20].

These superior traits have made DBS a common sampling method for gene screening and long-term genetic sample bio-banking. Within the last few years, DBS sampling has been used for clinical and pre-clinical pharmacokinetic studies, taking advantage of smaller sampling needs and simplified sample collection and handling. DBS sampling has also been used for disease surveillance in developing countries [21].

The presence of clinical samples which can be easily collected, stored, transported and archived is particularly important, especially in areas that do not have adequate infrastructure for processing of blood products. Moreover, the receipt of necessary amount of blood from newborns can be accompanied by many difficulties. The venepuncture is relatively invasive procedure that should be performed by trained staff, required materials and equipment; blood samples must be promptly processed and stored under controlled conditions. An analysis with the use of whole blood dried on filter paper can provide a viable alternative (Table 2).

Table 2: Comparison of blood collection methods (venipuncture and DBS) and advantage of DBS technology

No	Feature	Venipuncture	DBS
1.	**Remote sample collection**	No	**Yes**
2.	**Stable and transportable at ambient temperature**	No	**Yes**
3.	**Ease of use with minimal training required**	No	No
	Small collection volume		
4.	**Patient collection by finger stick**	No	**Yes**
5.	**Quick drying for enhanced sample stability**	No	**Yes**
6.		NA	**Yes**
	Protection from humidity and contamination		
7.	**Integrated collection and storage**	NA	**Yes**
8.		No	**Yes**

In recent years is increasing the application of DBS as method for seroepidemiological survey with focus viral infections: measles, mumps, rubella and hepatitis B virus. These four infections are vaccine-preventable diseases and are a major cause of morbidity and mortality in children worldwide. The high contagious index (>90% for measles and rubella, and >50% for mumps), the presence of heavy, leading to debilitating complications with high frequency and determine their health and socio-economic importance. Approximately 30% of reported cases of measles have one or more complications, such as disabling effects are most common in children under five. Reports from 2014 inform about 115,000 children (mostly under 5 years of age) deaths due to measles infection. This means that about 314 children die every day as a result of complications of this viral disease. The importance of rubella infection as a public health is determined by the teratogenic effects of rubella virus during pregnancy. There are a high percentage of miscarriages, stillbirths or congenital rubella syndrome. Mumps virus caused of meningitis and meningoencephalitis at about 10 to 30% of the infected and non-immunized against this virus. Hepatitis B virus acts the liver cells and is a major cause for the development of liver cirrhosis. According to data from 2000 reported cases of acute hepatitis B infection in the world are nearly 5.7 million, of which 521 000 - deaths. Adults, who suffered HBV, usually recover completely, but the infection can be a cause for the development of chronic carrier in infants and babies [22].

These viral infections weaken the immune system and open the door to secondary health problems such as pneumonia, blindness, diarrhea, encephalitis and etc. Children in poor areas of countries including malnourished and with uncontrolled personal and health care are particularly vulnerable to permanent damage, even with visible recovery. The severity and consequences of the above infections are determined mostly by the preliminary preparation of the immune system by vaccination and / or previous infection.

In 1998, the Regional Committee of the World Health Organization (WHO) for Europe defined as key and target objects of health policy nine vaccine-preventable diseases, including elimination of measles and reduce the incidence of congenital rubella syndrome (CRS) [23]. Even in the first decade of the 21st century, amid reached a low morbidity, outbreaks of measles and rubella are registered in several countries of Western and Central Europe. In Bulgaria 2009-2011, after long period without outbreaks (7 years) was developing one of the largest epidemics in the region - over 24,000 infected (infected - 24,365; morbidity - 324% ooo) and 24 died (mortality - 0.3% ooo, lethality - 0,1%) [24]. In the period 2011-2013, major measles outbreaks were registered in France, Ukraine, Georgia and Turkey. In 2012-2013 were reported a threefold increase of the incidence of rubella in Europe. In 2015 in the European region measles outbreaks were reported in Germany, Bosnia and Herzegovina, Serbia, France, Austria and Turkey (total number of infected were more than 7000), about rubella mainly in Poland with more than 2000 infected (nearly 86% of all registered by rubella in the region) and mumps in Poland, Great Britain, Spain, Germany, with more than 10,000 reported cases [22; 25].

According [26] measles, rubella and mumps are typical anthropology infections by clinical criteria for detecting and proving the disease are:

- Measles - generalized rash lasting more than three days, a temperature higher than 38^{o}C and one or more of the following symptoms: cough, runny nose, Koplik spots, conjunctivitis.
- Mumps - acute onset of unilateral or bilateral painful swelling of the parotid or other salivary gland, lasting more than two days.
- Rubella - acute onset of generalized maculopapular rash and arthralgia / arthritis, lymphadenopathy, or conjunctivitis.

The laboratory diagnosis is based on: detection of IgM antibodies or specific antibody response against measles, mumps or rubella in the absence of recent vaccination; detect the virus (non-vaccine strain) or viral nucleic acid in an appropriate clinical material. The collection of blood samples, particularly from children, is not always greeted with enthusiasm and maintaining a cold chain when transporting samples to the laboratory is not always achievable. Recently two approaches have been validated for use in the WHO Measles and Rubella Laboratory Network which have the potential to be useful tools for the measles/rubella programme; the use of dried blood spots and oral fluid samples. Dried blood spots have been used for a range of epidemiological studies as an alternative to serum. Antibody is stable in this form and so it is particularly valuable where the lack of a cold chain is an issue. This technique has recently been applied successfully to measles cases, and there is accumulating evidence that the technique will work as well for rubella cases [26].

Hepatitis B viral infection (HBV) that attacks the liver and can cause both acute and chronic disease. HBV is

transmitted through contact with the blood or other body fluids of an infected person. Although 95% of acute HBV infections occur in rescue, 5% of them pass into a chronic form [27]. The outcome of hepatitis B virus infection is characterized by the development of seroconversion and development of antibodies against surface viral antigen HBsAg (anti-HBs) and clears the virus from hepatocytes. In a small percentage of individuals accomplished seroconversion in serum and liver detect minor levels of HBV DNA, which are sometimes so low that cannot be detected with the available molecular tests, a condition described as occult or latent hepatitis B [28]. HBV reactivation is a clinically significant complication that can occur self-limiting, but in most cases it is acute and in 5% to 40% can lead to severe liver complications [29]. More than 686 000 people die every year due to complications of hepatitis B, including cirrhosis and liver cancer. The risk of developing chronic hepatitis B is greatest when virus infection occurs at an earlier age. 80–90% of infants infected during the first year of life develop chronic infections and 30–50% of children infected before the age of 6 years develop chronic infections.

The health system there are effective live (MMR vaccine) and recombinant (hepatitis B vaccine) virus vaccine, monovalent or combination, which provide 90-95% immunity against infectious agents. The main reason for the failure to control measles, mumps, rubella and hepatitis B virus is insufficiently vaccine coverage which creates the risk of disease on older age with more severe course and higher mortality. Despite the advantages of the vaccination it creates an opportunity for variability of wild viruses and reactions in the body that cause. Viral infections such as measles, mumps and rubella can have clinically and immunologically atypical reactions.

In recent years a growing and more affordable trips around the world, refugee crisis and anti-vaccination activities, increases the risk of formation of "landfill" from unprotected people and revival of considered endangered infections. In this regard scientific developments in order to enrich the knowledge of the viral nature and course of action are subject to the interest of many scientists.

In view of the widespread importance included in the current draft infections and the development of approaches (methods and equipment) in science, pressing need for alternative methods for the detection of measles, rubella, mumps and hepatitis B viruses. This would provide a foundation for further scientific developments in object other significant public health pathogens.

Collecting venous blood samples as a "gold standard" in the diagnosis of measles, rubella, mumps and hepatitis B virus, especially by children, is not always acceptable and often creates discomfort (pain, injection vacuum system, trained staff, maintenance of cold chain and etc.). This necessitates the search for alternative samples (dried blood spots and oral fluid) and techniques to detect of these viruses and specific antibody against them. In these samples viral agents and specific antibodies are present in stable form and can be archived, which is valuable in the absence of refrigeration circuit. The approaches are non-aggressive and more acceptable to the public (including young children, pregnant women, etc.) [30-33]. Sampling from remote, non-laboratory locations can be difficult due to the need for hours of air drying in a protected area and awkward handling with multiple components (i.e. lancets, filter paper cards, drying rack, and shipping containers). Additionally, proper application of blood to the filter paper requires training to reduce artifacts due to uneven sample coverage; touching the paper, too large or too small of a drop or too much time between drops can make a sample not

suitable for analysis. These difficulties have severely limited the ability of a patient to self-collect a sample from home or any remote location.

The optimizing of alternative approaches would provide valuable information in medical science and biology of viral infections. This line of research would be basis for future broader seroepidemiological survey on population level.

Since the opening of DBS technology at the beginning of the XX-th century up to now a number of research teams working in the direction of their wider application in various fields and improving the chemical structure of the used cellulose matrix. Currently it has developed a new generation of cards of filter paper (Whatman FTA elute cards) - acronym for rapid analysis technology serum samples and nucleic acids.

Uzicanin A. et. al. [34] and Helfand R. et. al. [35] 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.

Grüner and his colleagues 2015 provided protocol step by step [36-38] for use of DBS in immunological and molecular studies, comprising the following steps: (1) collecting blood; (2) preparation of blood spots; (3) drying the blood spots; (4) storage and transport; (5) elution of whole blood and/or serum from the DBS; and finally (6) an analysis of DBS eluates (Figure 1). This protocol is based on 1 762 related pairs serum/DBS for detection of hepatitis B - surface antigen (HBsAg), antibodies against HBV core antigen (anti-HBc), antibodies against HBV surface antigen (anti-HBs), HBV DNA, antibodies to hepatitis C virus (HCV) (anti-HCV), HCV RNA, and human immunodeficiency virus (HIV) 1-p24-antigen / anti-HIV 1/2 [36]. Subsequently, the protocol used in the pilot study "Drugs and Chronic Infectious Diseases" ("DRUCK Study"), which was conducted by the Robert Koch Institute in close cooperation with the National Reference Centre for hepatitis C [39].

3. Methods for collection and laboratory testing of DBS

Dried blood spots can be collected by pricking a finger or heel using sterile automatic lancets (1.5 - 2 mm). Blood stains will be stored on the cards of filter paper (Sample Collection Cards suitable for biological fluids, 4 rounds, and 13 mm), labelled and drying at room temperature for 30 minutes. The cards have the opportunity to collect 4 or 5 spots and each round can accommodate 75-80 μl of sample, they are ready for use, labelling, transport and archiving without the need freezing in a freezer or dry ice. Storage - ziplock bags with desiccant to 2^0C - 8^0C.

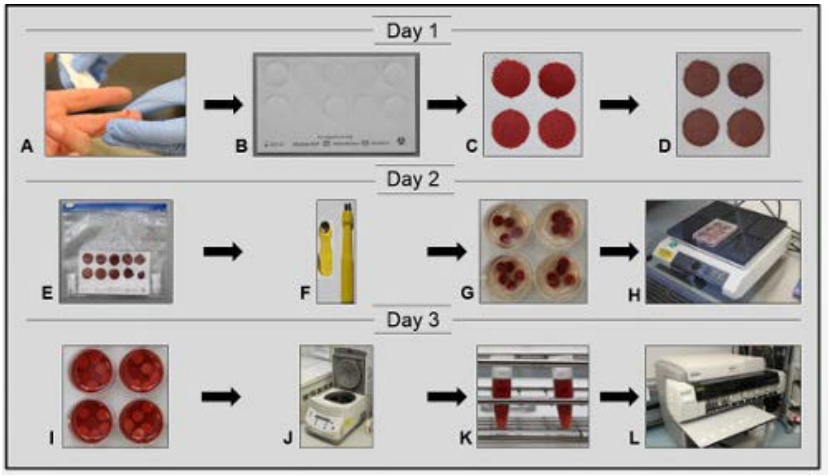
3.1. The necessary equipment is no specific

3.2. cards filter paper, rotary centrifuge, laboratory shaker, and freezer

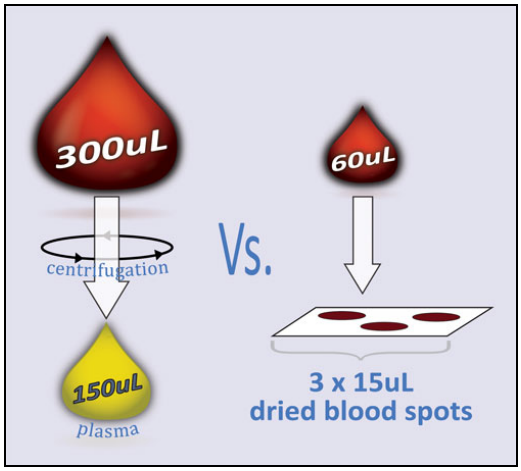
3.3. The extraction methods for elution of whole blood and serum from dried blood spots

The method for elution of whole blood and serum from dried blood spots has the following basic steps to work: (Figure 1):

- Punched single spot (DBS) with size 6 mm from each round of the cards of filter paper with the help of a perforating device. Transferring of all perforated dried blood spots from a patient in a single well of a 12-well plate.
- Add phosphate-buffered saline containing 0.05% Tween 20 and 0.08% sodium azide. Calculating the volume of the added buffer in accordance with the requirements of the analysis.
- Attaching the plate with the DBS and phosphate-buffered saline in a laboratory shaker and elution for a minimum of 4 hours.
- On the next day remove blood and haemolytic supernatant. Transfer of eluates in microcentrifuge tubes. Centrifuge for 2 minutes at 10 500 rpm, for the release of the supernatant and purifying the eluate.



(A)



(B)

Figure (1) Scheme of the extraction test procedure for the isolation of whole blood and serum from DBS (A) and advantage of DBS technology (B)

4. Conclusion

The DBS technique is optimized as an alternative approach (non-invasive, inexpensive, not requiring trained staff and cold chain for transport and storage) of venipuncture collection of clinical material in virology. This method facilitates the scientific researches about the concentration of virus specific antibodies in peripheral blood taken from a finger or heel; determining the percentage susceptibility / protection of the studied group of patients against vaccine-preventable infectious - measles, mumps, rubella and hepatitis B; social benefits - non-invasive technique for testing of small children and infants and applications in regions in the countries with not well developed logistics infrastructure.

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