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Dried blood spot sampling for detection of monoclonal immunoglobulin gene rearrangement

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ABSTRACT

Molecular methods are important tools for diagnosis and monitoring of many lymphoproliferative disorders. The reliability of lymphoma diagnoses is strikingly different between developed and developing countries, partly due to lack of access to these advanced molecular analyses. To overcome these problems, we propose a new application of dried blood spots (DBS) for detecting clonal B-cell populations in peripheral blood (PB).

We ensured that the DBS contained sufficient lymphocytes to perform a PCR-based clonality assay without producing false positives. Using the Namalwa B-cell line, we established that the assay is sensitive enough to detect 200 clonal cells in the analyzed sample. Very similar clonal results were obtained between DNA from DBS and fresh whole blood from patients with B-cell chronic lymphocytic leukemia. B-cell clonality can also be detected in DBS from African children with EBV-associated diseases.

This is the first study demonstrating that clonality testing can be performed on DBS samples, thus improving the diagnostic and monitoring options for lymphoproliferative diseases in resource-limited settings.

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1. Introduction

In many lymphoproliferative disorders, despite well-established histomorphological and immunophenotypical criteria, complementary molecular methods are required for conclusive diagnoses. According to published data, in about 10% of patients with suspected lymphoproliferative disorders, PCR-based clonality testing is useful in discriminating between reactive lymphoproliferation with polyclonally rearranged immunoglobulin or T-cell receptor genes, and malignant proliferation with clonal rearrangements [1–3]. Unfortunately, striking differences still exist in the reliability of lymphoma diagnoses between developed and developing countries. This is partly due to lack of access to these advanced molecular analyses, as occurs in several African countries in which lymphoma diagnoses are based on morphology alone.

In African countries, among lymphoproliferative disorders, of particular interest are those linked to Epstein–Barr virus infection (EBV). EBV is involved in the development of a wide range of B-cell malignancies, ranging from classical Hodgkin's lymphoma (HL) to non-Hodgkin's lymphoma (NHL), including Burkitt's lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), and immunoblastic lymphomas (IBL) in immunocompromised patients [4,5]. In Africa, primary infection with EBV occurs during infancy and early childhood. EBV-associated lymphomas are an important cause of mortality and morbidity in children, and BL in African children accounts for up to 75% of all childhood malignancies [6]. We have recently demonstrated that, in African children, NHLs are strongly associated with high levels of EBV in peripheral blood (PB) [7]. Notably, circulating malignant clones in the blood may be present in patients with late-stage lymphomas [8]. Indeed, although lymph nodes and bone marrow are the tissues of choice for detecting monoclonal populations in leukemias and lymphomas, PB has been shown to be reliable and safe in detecting malignant clones [9]. Thus, detecting clonality in the PB of patients with a high EBV load may represent a marker of tumor burden.

Clonality analysis in PB requires fresh whole blood and reliable transport to laboratories with specialized equipment. As such, an easy blood collection and conservation method would improve diagnosis in most cases. One option, to simplify collaboration between institutions in developed and developing countries, and/or between small laboratories and central facilities in the

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same country, relies on the use of filter paper for blood collection; dried blood spot (DBS) sampling is widely used in many types of tests, including chemical serological and genetic applications [10–12]. DBS ensures easy sample handling, transport and storage, especially for samples collected at remote sites where the laboratory equipment, personnel, or infrastructures necessary for correct handling of blood samples may not be available.

Here, we propose a new application of DBS sampling for detecting clonal B-cell populations in PB for the diagnosis and monitoring of B-cell malignancies.

2. Materials and methods

2.1. Blood, cell line and DBS samples

Blood was taken from five consenting healthy volunteer donors at the Instituto Oncologico Veneto, IOV-IRCCS, of Padova, Italy. The samples were tested to confirm lack of EBV-DNA. Six B-cell chronic lymphocytic leukemia (B-CLL) samples were referred to the IOV-IRCCS diagnostic laboratory for routine clonality testing, to complete the pathologist's diagnosis of B-CLL. All venous blood samples were collected in EDTA vacutainer tubes, and parallel samples were prepared: 50 μl of blood were spotted on Protein Saver TM 903 Cards (Whatman GmbH, Hahnestra, Germany), dried overnight at room temperature; 500 μl of whole blood were transferred to Eppendorf tubes for DNA extraction.

The Namalwa cell line, an EBV-positive BL line which is known to carry two integrated EBV type 1 genomes per cell [13], was established at the initial concentration of 10^6 cells/ml and diluted into whole blood from healthy donors to obtain 200–150–100–50–40–20–2–0 Namalwa cells per μl of blood. Fifty μl of each dilution was spotted on filter paper cards. All DBS samples were prepared in duplicate. A pellet with Namalwa cells only was used as a positive control of monoclonality.

DBS samples from African children with NHL and age-matched controls were collected at the Bugando Medical Centre in Tanzania; DBS cards were stored at room temperature in individual ziplock bags containing a desiccant, and sent to the IOV-IRCCS of Padova, Italy [7]. In this study, we analyzed 13 available samples.

2.2. DNA extraction

Genomic DNA was isolated with the automated MagNA Pure Compact instrument (Roche Applied Science, Indianapolis, IN). Briefly, 500 μl of fresh blood were processed according to the manufacturer's protocol contained in the MagNA Pure Compact Nucleic Acid Isolation kit I-Large Volume (Roche Applied Science), with elution volume set at 200 μl. For DNA extraction from DBS cards, one spot with 50 μl of dried blood was cut into pieces manually, avoiding cross-contamination. An external lysis step was performed in 180 μl of MagNA pure DNA tissue lysis buffer (Roche Applied Science) and 20 μl of proteinase K solutions at 56 °C overnight. After inactivation of proteinase K at 90 °C for 10 min and adjustment of the final volume to 500 μl with added phosphate-buffered saline (PBS), the DNA was extracted with the above protocol, with elution volume set at 50 μl, which is the minimum permitted by the protocol. The concentration of DNA extracted was determined on a spectrophotometer (NanoDrop ND-1000, Wilmington, DE). Approximately 11–24 ng/μl of DNA were recovered from the DBS cards.

2.3. Clonality assay

The extracted DNA samples were tested for B-cell immunoglobulin heavy chain (*IGH*) clonality with the *IGH* gene clonality assay (Invivoscribe Technologies, San Diego, CA), containing BIOMED-2 primers, positive and negative controls, in accordance with the manufacturer's instructions. As in specimens with limited DNA the recommended multiplex PCRs for suspected B-cell proliferation are the three framework subregions (FR) of the *IGH* gene, preferably followed by immunoglobulin kappa targets [14], the *IGH* FR1, FR2 and FR3 Master Mix was used, as well as the Specimen Control Size Ladder Master Mix for template amplification control. The combined use of a three FR multiplex strategy significantly improves clonality detection in mature B-cell malignancies, and the presence of clonal rearrangements can be detected in 89% of all B-cell diseases [15]. Each PCR reaction was prepared with the maximum volume of DNA provided in the protocol (5 μl) for DBS cards or 50 ng of DNA extracted directly from fresh blood. These post-PCR products were visualized by capillary electrophoresis on an ABI Prism 3730XL Genetic Analyzer, with subsequent analysis by ABI GeneMapper 4.0 software (Applied Biosystems). All samples were tested twice. A sample was considered to be clonal only when one or two reproducible peaks were detected within the valid range. Known non-specific peaks were excluded, in order to avoid false positives [1].

2.4. EBV-DNA quantification

EBV-DNA levels were quantified by real-time PCR assay, as previously described [7,16]. Briefly, a 106 base-pair *EBNA2* gene fragment is amplified with a primer pair (Fw 5′-CTG CCC ACC CTG AGG ATT TCC-3′ and Rv 5′-CTG CCA CCT GGC GGC AAC-3′) and an internal TaqMan probe (5'-FAM-AAT CCT CCT ACC CTC TCT TTA TGC CAT GTG TGT-TAMRA-3'). Each PCR was performed in a 25-μl reaction mix containing 5 μl of sample, 12.5 μl of LightCycler 480 Probes Master, 300 nM of each EBNA2 primer, and 100 nM of EBV type 1 probe. Amplification was carried out in a thermal cycler (LightCycler 480, Roche Diagnostics). A standard reference curve was obtained by five-fold serial dilution of an amplicon for EBV. The real-time PCR assay had a detection limit of 5 copies, with a dynamic range from 5 to 2×10^5 copies.

3. Results

3.1. Evaluation of polyclonal B cells on DBS

One of the limitations of DBS for laboratory diagnosis is the small quantity of sample. Since single-cell DNA can be amplified by generating a single (clonal) amplification product, a false positive result may occur if there are very few normal lymphocytes in the test sample. To rule out this possibility, although the BIOMED-2 protocol indicates that at least 100 ng of DNA should be used for each PCR reaction, we first verified whether 50 ng of DNA from normal whole blood (corresponding to about 7500 cells) was sufficient to detect a normal polyclonal pattern corresponding to B-lymphocytes carrying the different immunoglobulin gene rearrangements. DNA was extracted from 500 μl of whole blood from five donors, and 50 ng of each sample were analyzed for FR1-JH, FR2-JH, FR3-JH rearrangements of the *IGH* gene; a polyclonal pattern was observed in all cases. From these same donors, 50 μl of blood were spotted on DBS filters and 5 μl (about 50 ng) of the 50 μl DNA extracted were analyzed; polyclonal results were concordant with those obtained with DNA from fresh blood (Fig. 1). Thus, 5 μl of spotted blood contain sufficient lymphocytes to perform an *IGH* clonality assay without false positives.

3.2. Lower limit for detecting monoclonal IGH gene rearrangement on DBS

We assessed the lower limit of clonal detection in the PCR-based analysis of DBS samples with Namalwa B cells, diluted in the whole blood of a healthy donor; 50 μl of each dilution were spotted on DBS filters in duplicate. As Namalwa cells contain two integrated EBV genomes/cell [13], the number of Namalwa B clonal cells contained in samples diluted from DBS and analyzed for clonality was also verified by testing them for EBV-DNA copy number. The number of EBV copies was estimated by real-time PCR and the values were closely related to those expected ($r = 0.987$, $p < 0.0001$). DNA from 100% Namalwa cells (N) revealed a monoclonal *IGH* gene rearrangement in all three framework regions. With DNA from Namalwa cells diluted in normal blood, the highest sensitive clonal signal was obtained with primers amplifying sequences between the FR2-JH and FR3-JH regions. As shown in Fig. 2, a positive clonal signal could be detected in DBS samples containing 40 Namalwa cells per μl of blood. DBS samples with 20 or 2 Namalwa cells/μl revealed only a polyclonal pattern, similar to DBS spotted only with normal PB. Thus, clonal B-cell populations on DBS can be detected by PCR-based methods when represented by at least 200 clonal cells in the sample.

3.3. Clonality analysis on DBS from B-CLL and EBV-NHL

We tested the reliability of our approach for detecting clonality on DBS from patients with B-CLL. Equal amounts of DNA, either extracted from DBS or from fresh blood samples taken from 6 B-CLL patients, were analyzed by the same PCR-based approach. As shown in Fig. 3A, very similar profiles, with similar migration distances and heights of clonal peaks, were present in both DBS and fresh blood samples from each patient. We also analyzed DBS from 13 EBV-positive African children (ranging from 2525 to 35,506 EBV copies/ml of blood): 4 NHL and 9 controls. All samples were

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