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Development of Novel Paper-Based Assay for Direct Serum Separation

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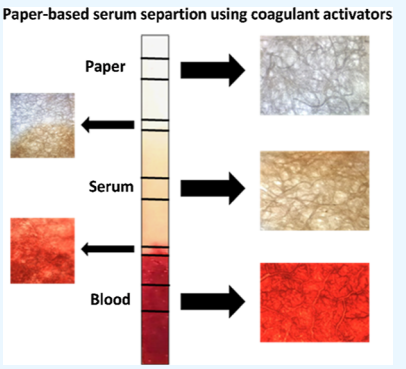
ABSTRACT: *Background*: Many conventional laboratory tests require serum separation using a clot activator/gel tube, followed by centrifugation in an equipped laboratory. The aim of this study is development of novel, equipment-free, paper-based assay for direct and efficient serum separation. *Methods*: Fresh blood was directly applied to wax-channeled filter paper treated with clotting activator/s and then observed for serum separation. The purity, efficiency, recovery, reproducibility, and applicability of the assay were validated after optimization. *Results*: Serum was successfully separated using activated partial thromboplastin time (APTT) reagent and calcium chloride-treated wax-channeled filter paper within 2 min. The assay was optimized using different coagulation activators, paper types, blood collection methods, and incubation conditions. Confirmation of serum separation from cellular components was achieved by direct visualization of the yellow serum band, microscopic imaging of the pure serum band, and absence of blood cells in recovered serum samples. Successful clotting was evaluated by the absence of clotting of recovered serum by prolonged prothrombin time and APTT, absence of fibrin degradation products, and absence of *Staphylococcus aureus*-induced coagulation. Absence of hemolysis was confirmed by undetectable hemoglobin from recovered serum bands. The applicability of serum separated in paper was tested directly by positive color change on paper using bicinchoninic acid protein reagent, on recovered serum samples treated with Biuret and Bradford reagents in tubes, or measurement of thyroid-stimulating hormone and urea compared to standard serum samples. Serum was separated using the paper-based assay from 40 voluntary donors and from the same donor for 15 days to confirm reproducibility. Dryness of coagulants in paper prevents serum separation that can be re-stored by a re-wetting step. *Conclusions*: Paper-based serum separation allows for development of sample-to-answer paper-based point-of-care tests or simple and direct blood sampling for routine diagnostic tests.



INTRODUCTION

Diagnostic laboratory procedures follow strict steps of sample collection, separation, and preparation before analysis of required target/s can be performed.[1] Most laboratory tests are carried out on the liquid portion of blood which require blood collection in specific tubes, followed by plasma or serum separation by centrifugation before detection of different analytes.[2,3] Serum is the liquid fraction of whole blood after clotting, which is considered the gold standard for many assays. Plasma is produced when whole blood is collected in anti-coagulant tubes. Accordingly, the major difference between serum and plasma is blood clotting.[2,3]

Blood clotting or coagulation is mediated by the intrinsic and extrinsic coagulation pathways that end by formation of thrombin and fibrin to form a meshwork of filaments with the aid of blood cells. Enhanced blood clotting within blood vessels under pathological conditions leads to thrombotic diseases, while delayed/deficient coagulation leads to bleeding disorders. Coagulation can be activated in vitro by addition of different coagulants to blood or by leaving fresh blood to rest for about 30 min. Coagulation assessment can be carried out clinically using many laboratory tests including clotting/bleeding time, the prothrombin time (PT) for assessment of the extrinsic and common coagulation pathways, activated partial thromboplastin time (APTT) for assessment of the intrinsic pathway deficiency, Kaolin clotting time for lupus anticoagulants, fibrinogen, D-dimer, and other tests for assessment of thrombosis.[3,4]

Advantages of plasma over serum are larger volume, no delayed clotting, less risk of hemolysis, the sample better represents the in vitro state of the patient, and significant reduction in the turnaround times.[3] Disadvantages of plasma over serum are the unknown effects of the anti-coagulant used on the assay and concentration is affected, as plasma collection has a general dilutional effect on biomarkers. Thus, serum provides a higher detection sensitivity compared to plasma.[3]

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Diagnostic standards have been established using a particular collection approach, which imposes the use of the same sample type in order to avoid improper diagnosis. For example, most clinical chemistry testing is performed using serum rather than plasma.[3,4]

Traditional serum collection procedures require central laboratories, electricity and centrifugation, and trained laboratory technicians. This process is complex, expensive, and time-consuming. Recent trends in blood plasma/serum separation show a rapid shift toward microscale processes and point-of-care testing (POCT). Development of simple, equipment-free POCT for serum separation is of great advantage.[5,6] A limited number of assays/devices for serum/plasma separation POCTs have been developed.[1,7] To the best of our knowledge, no paper-based assay for serum separation was reported. On the contrary, many paper-based POCTs were developed for plasma separation.[1,7,8]

Recently, a large number of studies have highlighted the wide applications of paper-based assays for sample collection and pre-treatment, disease detection and diagnosis, detection of microorganisms, food safety, environmental surveillance, monitoring and follow up, and drug testing for POCTs applications.[8−10] Current challenges of paper-based POCTs include development of sample-to-answer assays that incorporate blood collection, separation, and pre-treatment techniques with target detection assays.[1,9,10] This study is the first to separate serum using simple treated microfluidic filter paper with a variety of "coagulants" as a pre-analyte targeting requirement for POCT of biomarkers when sensitivity is critical. The efficiency, purity, reproducibility, and applicability of this approach were investigated.

MATERIALS AND METHODS

Hydrophobic Channels Fabrication on Filter and Chromatography Paper. Fabrication of hydrophobic wax channels and patterns on filter and chromatography paper was performed using a Xerox 8580 solid ink printer, followed by heating with a laminator device.[8] Different filter papers were tested for serum separation using the membrane filtration approach including Whatman qualitative filter paper grades 1, 3, 5, 6, and 598 with particle retention liquid of 11, 6, 2.5, 3, and 8−10 μm, respectively, and Whatman quantitative ashless filter paper grade 42 with particle retention liquid of 2.5 μm and chromatography paper (Whatman, USA).

Blood Coagulation and Serum Separation. Different coagulation activators were tested to separate serum directly on paper including PT reagent, APTT reagent, calcium chloride ($CaCl_2$) 0.025 mol/L (International Medical Diagnostic, Diamond, Zarqa, Jordan), recombinant thromboplastin reagent (Spectrum, Egypt), Cephalin, and Kaolin (BIO-CK, BIOLABO SA, France). PT reagent usually contains thromboplastin composed of a tissue factor mixed with synthetic phospholipid. APTT reagent contains activated cephaloplastin, a phospholipid preparation derived from the rabbit brain with ellagic acid as an activator.[3,4] Kaolin is aluminum phyllosilicate clay mineral with negative charge particles that activate the intrinsic pathway. $CaCl_2$ is an inorganic salt that enhances blood coagulation and usually used with APTT reagents.[3,4] Coagulation activators (PT reagent, APTT reagent, $CaCl_2$, Cephalin, and Kaolin) are stored at 2−8 °C, and the reagents are stable until the expiry date within 2 years according to the manufacturer. 5 μL of different coagulation reagents was added to wax-channeled paper, followed by addition of 5 μL of whole blood from finger prick or plain capillary tube. The reaction was allowed for 2 min, and serum separation was observed directly by naked eye or under the microscope (40× light compound microscope, LEICA DM2500, Singapore), and images were taken by a digital color camera (LEICA MC170HD digital color camera, LEICA microsystems, Singapore). Assay optimization under different conditions including filter/chromatography paper type and grade, coagulation reagent type/concentration/volume, blood collection method, reaction time, and wax channel design and size were investigated. Fresh whole blood samples were collected from 40 voluntary donors after providing formal consent. Dryness and storage conditions of coagulation activators on paper were tested at room temperature (RT), dried and re-wetted with Phosphate-buffered saline (PBS), NaCl buffer, or distilled water, sealed in closed cassette with silica, or by addition of 10% glycerol with 0.1% tween 20, and compared to standard testing with wet fresh refrigerated reagents under similar conditions over days. The effect of storage time was assessed by measuring the length of serum bands over days of storage and compared to day 1 under the same conditions and presented as a percentage.

Hematological and Clotting Assessment of Paper-Based Separated Serum. Recovered serum was obtained by cutting pieces of filter paper containing separated serum bands avoiding red blood cells (RBCs) region and inserted into microcentrifuge tubes containing 100 μL of normal saline (0.9%) and then incubated in shaking water bath (GFL, Germany) at 37 °C for 1 h. Standard determination of PT and APTT on recovered serum separated on paper or serum separated using standard collection methods was carried out using manufacturer instructions by a coagulometer (BIOBASE, China). Fibrinogen and D-dimer testing was carried using manufacturer instructions in a reference laboratory (Stago, France). The collective blood count was determined using a hematological analyzer (Sysmex, USA). The *Staphylococcus aureus* (*S. aureus*) coagulase test was performed, as detailed in ref 11. Briefly, equivalent concentrations of characterized *S. aureus* strains were mixed with recovered serum separated by paper, and traditional serum or plasma samples, and then observed for the clotting using slide test for 15 min and tube test for 24 h.

Testing Paper-Based Isolated Serum for Total Proteins, Urea, and TSH Compared to Standard Serum Assays. Functionality of the serum separation band using filter paper to detect serum proteins was tested by direct addition of 5 μL Biuret reagent (GCC, UK) and observed for violet color development as positive reaction, addition of 5 μL Bradford reagent (abcam, UK) with positive reaction indicated by blue color formation, or by addition of 5 μL of Pierce (bicinchoninic acid) BCA protein assay reagent (Pierce, USA) with positive reaction indicated by purple color development. Furthermore, recovered serum from paper was withdrawn into new microcentrifuge tubes, followed by addition of 100 μL Bradford reagent, 200 μL of Biuret reagent, or 100 μL Pierce BCA, thyroid stimulating hormone (TSH) kit reagent (DiaMetra, Italy), and urea Berthelot reagent (Beacon diagnostics, India) and then observed for color changes compared to standard serum samples under similar conditions. Quantitative measurement of TSH was carried by the ELISA kit (DiaMetra, Italy) according to manufacturer's instruction and as described in detail in ref 8. Quantitative measurement of

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urea was carried out in a reference laboratory according to standard operating procedures.

RESULTS

Paper-Based Serum Separation Using Different Chromatography/Filter Papers. Whole fresh blood (5 μL) was added directly to channeled chromatography paper and filter paper of different grades and characteristics treated with 5 μL APTT reagent plus 5 μL $CaCl_2$ and observed for serum separation (yellow band) (Figure 1). No serum bands

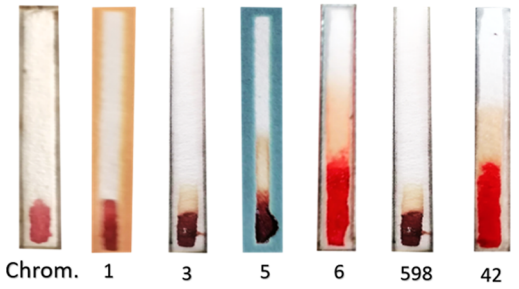

Figure 1. Serum separation on chromatography paper (Chrom.) and filter paper grades 1, 3, 5, 6, 598, and 42.

can be observed with chromatography paper or filter paper grade 1; small serum bands can be observed with filter paper grade 3 and 598, while larger bands can be observed with grades 5, 6, and 42. Filter paper grade 42 was used for further experiments.

Paper-Based Serum Separation Using Different Coagulation Activators. Different coagulation activators were tested for their ability to induce serum separation in paper including Kaolin Cephalin, thromboplastin, PT reagent, APTT reagent, and $CaCl_2$ (Figure 2). The ability of Kaolin Cephalin, thromboplastin, and PT reagents to induce serum separation was limited, generating small serum bands (Figure 2A), even after doubling the volume of the reagents for coagulation enhancement (Figure 2B). APTT reagent plus $CaCl_2$ induced better serum separation and larger bands (Figure 2C) compared to $CaCl_2$ alone (Figure 2E,F). Decreasing the volume of APTT reagent plus $CaCl_2$ to the half also decreased the serum band size (Figure 2D). 5 μL of APTT reagent plus 5 μL of $CaCl_2$, followed by addition of 5 μL of blood, were used for further analysis.

Paper-Based Serum Separation Using Different Donors and Blood Collection Methods. To test for donor-to-donor variability, 17 blood samples were tested using the paper-based serum separation by application of 5 μL of APTT reagent plus 5 μL of $CaCl_2$, followed by addition of fixed fresh blood volumes by micropipette (5 μL) (Figure 3A) or addition of one blood drop using finger prick without volume control from 17 donors (Figure 3B). Serum was successfully separated from all donors with minor variability in serum band size (Figure 3A,B). This would indicate the applicability of paper-based serum separation on different blood samples using direct finger prick.

Purity of Paper-Based Serum Separation Using a Light Compound Microscope. To confirm successful separation of serum in paper, 5 μL of APTT reagent plus 5 μL of $CaCl_2$ were added to channeled filter paper grade 42, followed by the addition of 5 μL fresh blood. The blood within the channeled paper was then observed under a light compound microscope (40×), and images were taken using a digital color camera attached to the microscope (Figure 4). Agglutinated RBCs can be observed (red color), followed by a small transition zone of blood and serum (mixed red and yellow color) and a pure serum band (yellow color) where no RBCs can be observed. Original white paper color can be noticed at the end (Figure 4).

Confirmation of Separation of Serum and Removal of Blood Clot. To confirm the ability of paper-based assay to separate serum rather than plasma, serum was simultaneously separated from the same donor using paper-based separation

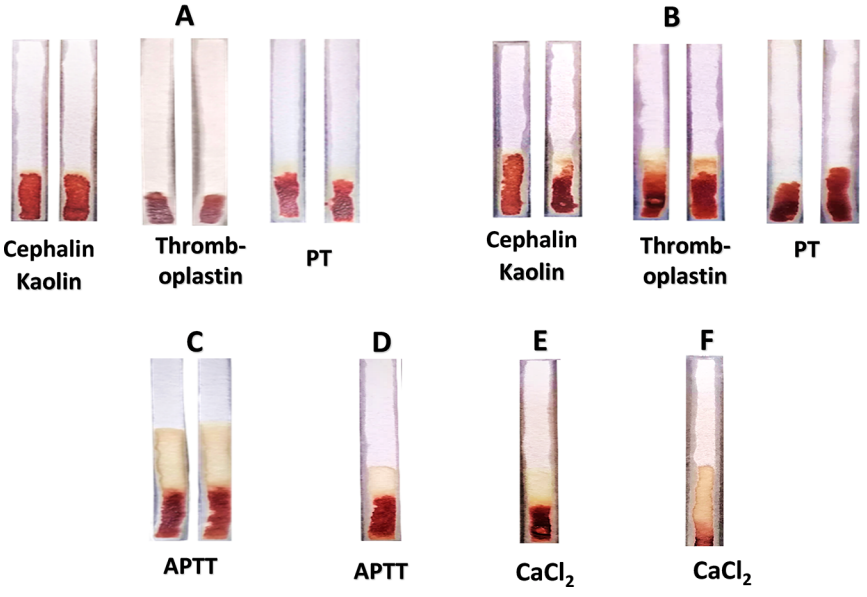

Figure 2. Serum separation on wax-channeled filter paper grade 42 treated with different coagulation factors, followed by addition of equal volumes of whole fresh blood (3 μL). (A) 3 μL of Cephalin Kaolin, thromboplastin, and PT reagents in duplicates. (B) 6 μL of Cephalin Kaolin, thromboplastin, and PT reagents in duplicates. (C) 3 μL of APTT reagent plus 3 μL of $CaCl_2$ in duplicate. (D) 1.5 μL of APTT reagent plus 1.5 μL of $CaCl_2$. (E) 3 μL of $CaCl_2$ only. (F) 3 μL of blood, followed by addition of $CaCl_2$ only.

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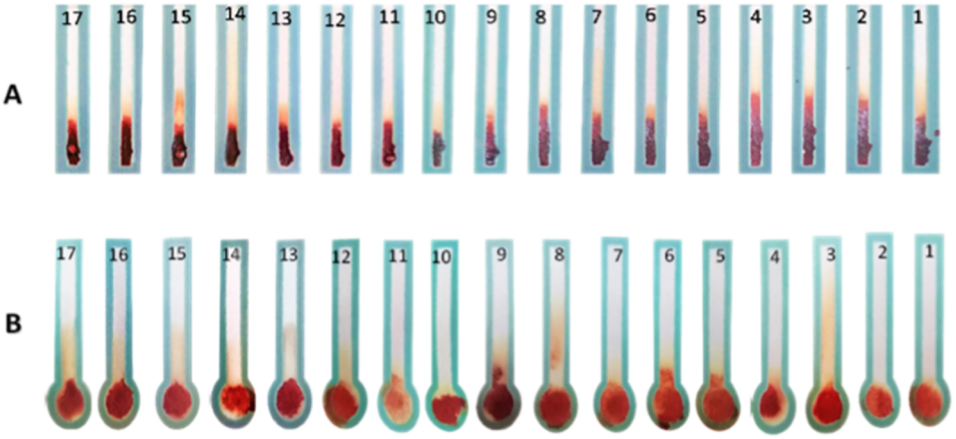

Figure 3. Paper-based serum separation using different donors and different blood collection methods. 5 μL of APTT reagent plus 5 μL of $CaCl_2$, followed by addition of 5 μL blood from 17 donors (A) or addition of one drop of blood collected directly by finger prick (B). Blood donors and channel design were different in panel (A) compared to panel (B).

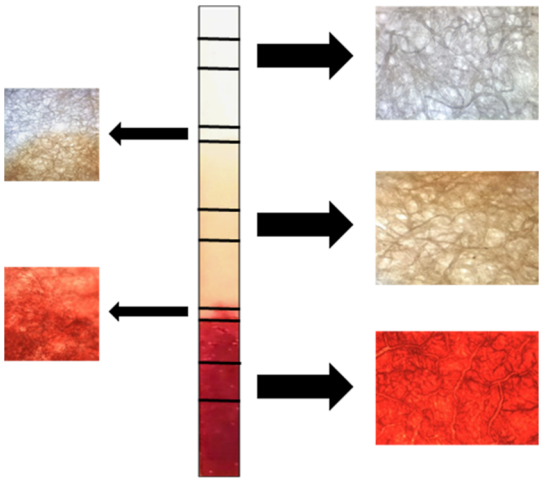

Figure 4. Purity of paper-based serum separation using a light compound microscope (40×). 5 μL of APTT reagent plus 5 μL of $CaCl_2$, followed by addition of 5 μL fresh blood on channeled filter paper grade 42.

and standard collection by clot activator tubes and centrifugation, while plasma was separated from the same donor using citrated tubes and centrifugation. As shown in Table 1, PT, APTT, fibrinogen, D-dimer, and *S. aureus* coagulase test readings of standard plasma samples were within the normal range. All readings of serum samples (paper-based and standard) were not recordable due to the absence of clotting factors, indicating successful clot formation by both methods (paper-based and standard) (Table 1). In addition, collective blood count analysis on recovered serum from filter paper compared to standard serum/plasma indicated the absence of all blood cells (RBCs, WBCs, and platelets), which further confirms the successful separation of blood clot and blood cells in paper. Absence of hemolysis can be noted by the absence of hemoglobin (Hb) in all samples (Table 1).

Qualitative determination of analytes using paper-based serum separation was compared to that using standard tube collected serum. The functionality of paper-based serum separation assay for qualitative and semi-quantitative determination of serum proteins directly on paper or using recovered samples was tested to confirm efficient wicking of serum proteins. BCA protein detection reagent was added directly to serum bands on paper, and successful detection of serum proteins was indicated by purple color transformation compared to negative reaction with original green color (Figure 5).

Additionally, Bradford and Biuret reagents were added to paper-separation recovered sera and to conventional tube collected sera and observed for color change (Figure 6). Blue color transformation indicated successful detection of proteins by Bradford reagent and violet color for Biuret reagent compared to negative control (Figure 6A,B). To test the applicability of paper-based serum separation to measure targeted analytes compared to standard tube separation, TSH was measured in recovered sera from paper, standard sera, or negative control using ELISA in triplicates. As shown in Table 2, TSH concentration was comparable between paper-based serum samples (1.05 mIU/L) and standard sera (1.2 mIU/L). Similarly, the urea concentration obtained from paper-based serum samples (24 mg/dL) was equal to that obtained from standard samples (25 mg/dL).

Effect of Dryness and Storage Conditions of Coagulation Activators in Paper-Based Assay. The coagulation activators (APTT reagent and $CaCl_2$) were tested

Table 1. Coagulation and Hematological Assessment of Serum Separated by Filter Paper Compared to Standard Samples[a]

sample	PT	APTT	fibrinogen	D-dimer	S. aureus coagulase	RBCs	WBCs	platelets	Hb
serum (standard method)	ND	ND	ND	ND	ND	ND	ND	ND	ND
serum (paper-based method)	ND	ND	ND	ND	ND	ND	ND	ND	ND
plasma (standard method)	7.6 ± 2.3 s	35.9 ± 6.8 s	3.4 ± 1.2 g/L	145 ± 75 ng/mL	positive	ND	ND	ND	ND

[a]Serum or plasma separated using standard methods or using paper-based assay were tested for PT, APTT, fibrinogen, D-dimer, and blood count for RBCs, WBCs, platelets, and Hb. PT: prothrombin time, APTT: activated partial thromboplastin time, *S. aureus*: *Staphylococcus aureus*, RBCs: red blood cells, WBCs: white blood cells, Hb: hemoglobin, ND: not detected (mean ± SD, n = 3).

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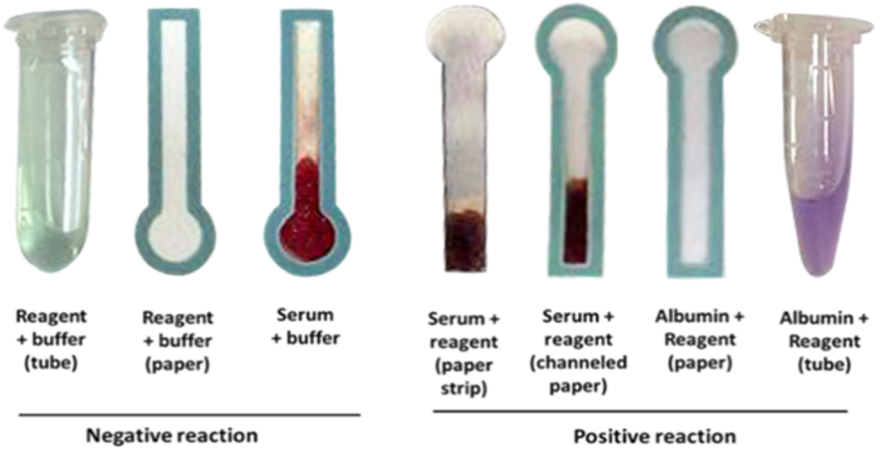

Figure 5. Protein detection directly on serum obtained using paper assay. BCA protein reagent added to normal saline (buffer) in tube or in paper (negative reaction) or added to serum separated in channeled or strip paper (positive reaction). Positive control included BCA reagent added to albumin directly on paper or in tube.

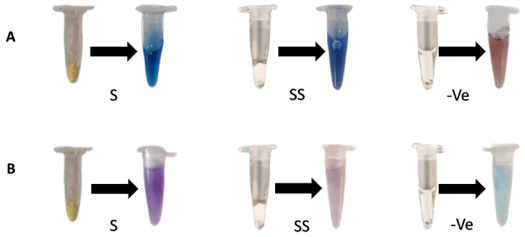

Figure 6. Protein detection from recovered samples of serum obtained using paper assay or standard method. (A) 100 μL of Bradford reagent was added to serum obtained by the standard method (S), recovered serum from paper (SS), or negative control using buffer (−ve). (B) 200 μL of Biuret reagent was added to standard serum (S), recovered serum from paper (SS), or negative control (−ve).

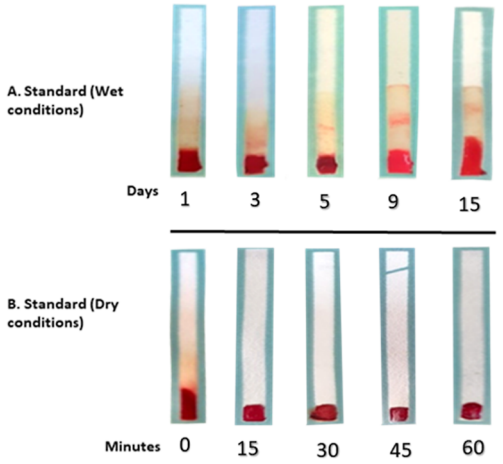

Figure 7. Effect of pre-dryness of coagulation activators in paper on serum separation. (A) APTT reagent and $CaCl_2$ adsorbed to paper, followed by addition of blood while the reaction zone is still wet and repeated over 15 days. (B) APTT reagent and $CaCl_2$ adsorbed to paper and left to dry for the indicated time in minutes, followed by addition of blood.

under "standard conditions" using fresh refrigerated reagents brought to RT and then adsorbed to channeled paper, followed by addition of a blood sample immediately while the reaction zone is still wet. Repeating the same procedure daily under the same conditions (same coagulation reagents and volumes, filter paper, channel pattern, donor, etc.) for 15 days produces consistent serum separation confirming the assay reproducibility (Figure 7A). However, once the coagulation reagents start to dry within the filter paper at RT, it lost the ability to separate serum within 15 min (Figure 7B).

To enhance the applicability of the assay for POCT with minimal requirements, coagulation activators were left at RT and tested daily under similar conditions. Serum separation can be observed for up to 15 days, indicating that reagents remain functional without refrigeration (Figure 8A). Furthermore, if coagulation reagents adsorbed to paper and then sealed and left at RT or refrigerated and tested daily for 15 days by re-wetting with distilled water, followed by addition of blood sample, serum separation occurs efficiently in both methods, confirming the important role of wet conditions rather than refrigeration (Figure 8B,C). Addition of 10% glycerol and/or 0.1 tween 20, sealing, or plastic cassettes packaging to preserve the activity of coagulation agents in dry conditions failed to induce serum separation (Figure 8D−F). The efficiency of serum separation on day 15 of storage was about 85% compared to initial values (serum band length at day 15 was 6 mm compared to 7 mm at day 1).

Table 2. TSH and Urea Measurements of Recovered Serum From Paper or Standard Assay on the Same Donor[a]

sample	TSH OD reading	TSH concentration mIU/L	urea concentration mg/dL
serum (standard method)	0.136 ± 0.009	1.05	25
control (filter paper with normal saline only)	0.01966 ± 0.0009	0	0
serum (paper-based assay)	0.028 ± 0.002	1.2	24

[a]TSH: thyroid stimulating hormone. OD: optical density.

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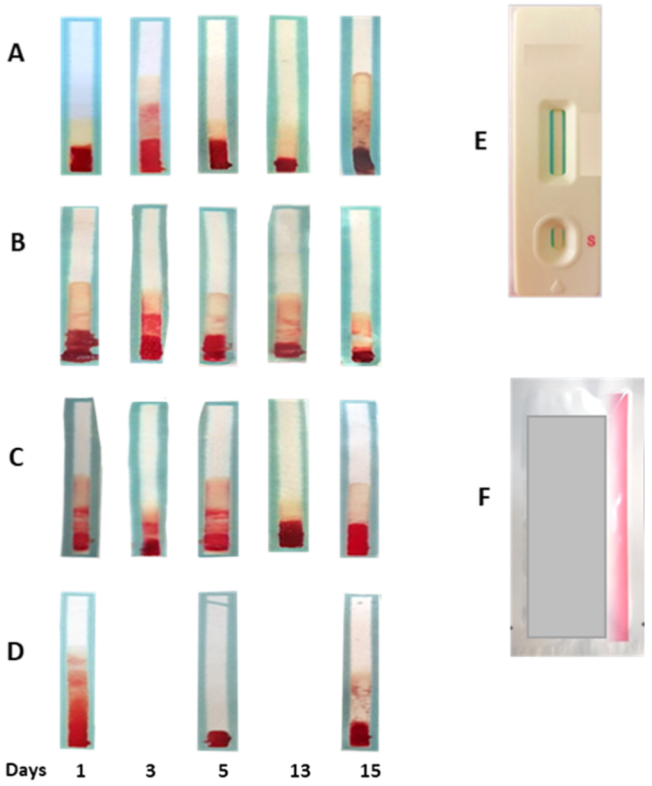

Figure 8. Effect of storage conditions of coagulation activators in paper. (A) APTT and $CaCl_2$ reagents stored at RT for the indicated days and then added to paper, followed by addition of blood. APTT and $CaCl_2$ reagents adsorbed to paper, sealed and left at RT (B), or refrigerated (C) for the indicated days, then re-wetted with distilled water, followed by addition of blood. (D) Glycerol and tween 20 mixed with APTT and $CaCl_2$ reagents and then adsorbed to paper and left at RT for the indicated days, followed by addition of blood. (E) Plastic cassette and (F) packaging used for assay storage.

DISCUSSION

A majority of clinical laboratory tests are carried on the liquid portion of blood: either serum or plasma. The presence of cellular components or their hemolysis products in serum/plasma complicate laboratory tests, interfere with measurement methods, change the color of the fluid, and decrease test accuracy.[2,3] POCTs also require sample pre-treatment and separation for similar reasons.[1,7] The main differences between serum and plasma are that the bulk of fibrinogen and clotting factors have been removed from serum, the concentrations of proteins, immunoglobulins, complement factors, enzymes, lipids, metabolites, nucleic acids, drugs, and ions are different, and the dilutional and biochemical effect of anti-coagulants versus clot activation and removal. Therefore, serum and plasma are not interchangeable specimens and serum collection and separation is mandatory for many diagnostic tests.[2,3]

The need for specific tubes, large volume of blood, equipment, electricity, and trained technicians complicates routine blood collection and separation with increased time and cost.[6] POCT would be an ideal solution to these limitations.[6,12] Paper-based microfluidic analytical devices (μPADs) fit themselves into POCT with in-demand characteristics like self-driven flow, high surface to volume ratio, ubiquity, biocompatibility, natural disposability, portability, accessibility, flexibility, and low cost.[1,8−10] In this study, we developed a simple, direct, equipment-free paper-based serum separation assay. Blood clot was activated using APTT and $CaCl_2$ reagents, and separation was achieved passively through paper filtration and capillary absorption. The assay is useful for different paper-based POCT, serum collection in remote or resource limited areas, emergency and military situations, long-term serum storage, and frequent follow ups.[6]

Generally, if fresh blood is collected without anti-coagulants and left to rest for a proper time, it would coagulate naturally. Addition of clotting agents would significantly enhance the process in terms of efficiency and time.[4] Natural blood clotting is not applicable to direct addition of blood to paper substrates or any microfluidic devices as immediate wicking of plasma would occur due to capillary force before proper clotting is allowed. The wicking microfluidic approach or filtration is more appropriate to separate blood plasma rather than serum.[6] Furthermore, cellular aggregates or blood clots will form fouling layers in paper/microfluidic channels, which prevent further blood separation and decrease the overall yield.[5] Accordingly, many paper-based assays for plasma separation were reported compared to limited assays for serum separation.[1,7,8]

Few studies proposed serum separation using paper or membranes by application of fresh blood.[5,6,13] The actual separation of serum rather than plasma and the purity of serum/plasma was not confirmed. Separated plasma or serum bands look similar by the naked eye or under the microscope and can be distinguished only by further testing to confirm clot formation and removal. These assays require complex devices, materials, or design including a pulling-force spinning top, using a polycaprolactone-filled glass microfiber membrane, or "High Yield Passive Erythrocyte Removal" (HYPER) technology composed of three layers.[5,6,13] Furthermore, while blood clotting occurs naturally for fresh blood, this is a slow and weak process complicated by cellular hemolysis and release of intracellular components.[2,3] Two studies used microfluidic paper-based lateral flow assay consisting of sample pad, analytical cellulose or nitrocellulose membrane, and wicking pad for blood coagulation screening and anti-coagulation therapy monitoring.[14,15] Other studies have used different methods for targeted separation, focusing, extraction, or concentration of proteins, nucleic acid, or ions from blood in paper-based assays using chemical modification, electrical current, pH gradient, and other methods.[1,16−18] These techniques are useful for specific purposes only, require extra force/power or chemical treatment for separation, and are complex, expensive, and time-consuming.[1]

In this study, coagulation was activated within paper channels using APTT and $CaCl_2$ reagents known to activate the coagulation cascade efficiently and rapidly. APTT reagent activates the intrinsic pathway of coagulation, while recalcification with calcium chloride leads to formation of a stable fibrin clot.[3,4] Confirmation of successful blood clot formation and pure serum separation was carried out by microscopic imaging, absence of clotting by prolonged PT and APTT time, absence of fibrin and D-dimer, absence of *S. aureus* coagulation, and absence of blood cells or hemoglobin on paper-based serum separation.[4,8,11] The paper substrate provides direct separation of the liquid portion through capillary absorption without the need for external force.

The functionality and applicability of serum separated in paper were confirmed directly by positive color change on paper using BCA protein detection reagent, on recovered

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paper-based serum samples treated with Biuret and Bradford protein detection reagents in tubes and by measurement of TSH and urea compared to standard serum samples. Accordingly, paper-based separated serum can be used for development of paper-based POCTs or recovery of samples for further analysis. Other studies have illustrated the applicability of paper-based assays for analyte detection using traditionally separated serum or plasma.[1,9,10,13]

The advantages of the current assay include a simple direct separation of serum in one step by adding fresh blood to the reaction zone that can be performed by lay personnel. Passive adsorption of commonly used clotting activators on non-modified, striped, or wax-patterned filter paper further simplifies the assay manufacturing and applicability. The assay requires a small volume of blood (about 5 μL) that can be applied directly by finger prick. The total time required for the reaction is limited to a few minutes. The assay is cheap, equipment-free, efficient, reproducible, and applicable. Paper-based separation of serum could be an alternative to traditional serum separation procedures applied by diagnostics laboratories. Furthermore, it would be helpful for the expanding number of paper-based POC applications without the need for pre-treatment steps. Separated serum using paper could be used for long-term storage of serum samples. The dilutional effect of APTT and $CaCl_2$ reagents on the obtained serum and/or the dilutional effect of buffer used for serum recovery from paper require correction and need further standardization in future.

The innovative aspects of paper-based assay for serum separation include combining the efficiency of coagulant activators with the porous structure of paper within a microfluidic channel to achieve fast, efficient, microscale, passive, and directional serum separation. The coagulant activators achieve potent clotting within seconds, the porous structure of paper traps blood clot, the capillary action mediates passive wicking of serum band, while the microfluidic channel can direct flow of serum to other reaction zones to perform analyte testing, facilitating the sample-to-answer process.

No other reports have combined the use of coagulants, paper, and microfluidic channels for serum separation. Other studies reported serum separation POCTs including using pulling-force spinning top combined with paper-based assay for COVID-19 diagnosis,[13] HYPER technology that utilizes capillary forces, and crossflow filtration for the separation of whole blood combined with paper-based systems for iron and vitamin A detection.[5] A 3D membrane-based microfluidic device of polycaprolactone-filled glass microfiber was used to separate blood plasma/serum and facilitate downstream assays.[6] Blood coagulation screening using nitrocellulose membrane and lateral flow demonstrate RBCs travel distance in a given time is related to the blood clotting time using different $CaCl_2$ concentrations.[14] Digital image analysis of the RBC travel distance was used to measure the feasibility of using this device for anti-coagulant monitoring among patients compared to controls.[15] The blood sampling device using a grooved, asymmetrical, polysulfonate membrane with sucrose was developed for efficient serum separation.[19] Other POCTs reported separation of plasma rather than serum using a different approach.[7,8]

Dryness of APTT and $CaCl_2$ reagents in paper leads to the loss of its coagulation ability within minutes. This is likely because the reaction required wet conditions to allow proper coagulation to occur before wicking of blood happens or due to decrease wettability and absorption ability of paper. A similar effect was noted with antibody-treated paper.[20] This limitation can be overcome by re-wetting the paper that contains the coagulation activators with buffer or distilled water. Elution with buffer is commonly used to distinguish agglutinated from non-agglutinated blood in paper.[20,21] Other studies have developed different methods to enhance paper wettability after treatment including plasma treatment and nanoparticle addition.[20,22]

Coagulation activators are usually stored at 2−8 °C requiring refrigeration. APTT and $CaCl_2$ reagents adsorbed to paper and left at room temperature retain functionality for up to 2 weeks. The fibrous structure of paper preserves and protects biochemical molecules, is biocompatible, and is biomolecule-friendly.[1,5,8,10] Addition of polysaccharides like chitosan, sugar-like glycerol, surfactants like tween 20, and bovine serum albumin has been shown to enhance antibody survival in paper.[8,23,24] Preliminary attempts to enhance coagulants survival in paper with glycerol and tween 20 failed in this study mostly due to differences in chemical composition, mechanism of action, and dryness effect. Proper sealing and packaging and addition of preservatives are likely to extend the shelf-life of the assay. The functionality of the paper-adsorbed clotting activator (APTT and $CaCl_2$ reagents) for long-term use under ambient conditions needs further analysis. Patients with bleeding disorders or patients treated with anti-coagulants therapy might require longer time for blood clotting, and therefore, modification of clotting activator/agent or reaction time in paper-based assays might be needed.

In this study, storage of paper-based assay was functional for 15 days with retained efficacy of 85% compared to fresh samples. Similarly, serum components were maintained at ≥85% of initial values after 10 days of storage using a polysulfonate membrane impregnated with sucrose for separation.[19] Saidykhan et al. (2021) developed a paper-based device for measuring fibrinogen in blood plasma using wax-printed chromatographic paper strips modified with an immobilized thrombin reagent. This study reported that storing strips in aluminum bags with a desiccant in a fridge or freezer retains strip activity and functionality for 3 weeks. On the other hand, storing strips at ambient temperature gave acceptable measurements after the first week only, as increased desiccation might increase paper and reagent hydrophobicity. The addition of stabilizers, excipients, and processing techniques such as lyophilization can help store thrombin activity and enhance storage efficacy.[25] The assay used by Hegener et al. was stored in controlled room temperature of 20−22 °C and was used successfully within 3 months of production.[15]

Multiple recent reviews have highlighted the promising value of paper-based, microfluidic, and lab-on-a-chip technologies for POC diagnosis and monitoring of hemostasis and coagulation.[26−29] Many paper-based devices were developed to assess whole blood coagulation,[30,31] plasma fibrinogen concentration,[25,32−34] detection of thrombin,[35] and anti-coagulation therapy monitoring.[14,15] A microfluidic sensor named "ClotChip" was developed for POC assessment of hemostasis.[36,37] Another microfluidic chip coated with PT reagent was developed for blood coagulation assessment.[38] A microfluidic paper analytic POC device was used for monitoring of direct thrombin inhibitors.[39] A bio-microfluidic

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device with Raman spectroscopy, portable optical blood coagulation detector, and polymethyl methacrylate-based smart microfluidic devices were used for POC testing of PT.[40−42] Recently, printed circuit board digital microfluidics and blood viscosity monitoring using droplet microfluidics were used for POC blood coagulation assays as well.[43,44]

CONCLUSIONS

A novel, paper-based serum separation assay was developed using clotting activators passively adsorbed to wax-channeled filter paper, followed by direct addition of fresh whole blood. The assay was thoroughly optimized. The paper-based assay clotting efficiency and purity were proved using visual detection of the yellow serum band, microscopic imaging, absence of coagulation markers, absence of *S. aureus* coagulation, and absence of blood cells and hemoglobin. The assay reproducibility was tested successfully on 40 voluntary donors or on the same donor for 15 days. The assay applicability was confirmed directly on serum separated by paper using color intensity of plasma proteins and on recovered serum samples by measurement of TSH and urea concentrations compared to standard serum samples. Serum proteins, TSH, and urea are important clinical tests used for diagnosis of thyroid and kidney diseases. This assay is cheap, simple, fast, and reliable, requires a small volume of blood, and would enhance the applications of paper-based POCTs. Elution of serum samples collected using paper can be used for further diagnostic laboratory analysis pending further standardization.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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