Point of care diagnosis of dry eye disease with a sensitive immunoassay for dual biomarker detection

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ABSTRACT

Dry eye disease (DED) is a multifactorial eye disease with few effective methods for clinical diagnosis and treatment. It is the most common eye disease with significant health challenges of the unprecedented aging population. Recent proteomic studies and clinical research have led to the discovery of several biologically relevant biomarkers, with increased levels of Interleukin-6 (IL-6) and decreased levels of lactoferrin being clinically validated in the progression of DED. In this study, a sensitive point of care (POC) DED diagnostic method was developed for targeting dual biomarkers of IL-6 and lactoferrin in the tear samples. A paper-based lateral flow immunoassay (LFIA) was established in a double-antibody sandwich fashion with colloid gold nanoparticles acting as probes. The minimal detection concentrations were 0.1 ng/ml and 10 ng/ml for IL-6 and lactoferrin, respectively. Separated conventional ELISA tests were also performed with data confirming results from the LFIA tests. A trial study was conducted with 20 tear samples from DED patients and healthy controls. All DED tear samples exhibited significantly higher levels of IL-6 and decreased levels of lactoferrin, as compared to the normal controls. A quantitative analysis of LFIA images was carried out using ImageJ software for an accurate data interpretation. This dual biomarker detection method is sensitive and affordable with quick turnaround time. Design of a larger clinical study in the future can further validate this POC assay for early diagnosis as well as patients’ self-management of chronic states of DED.

Keywords:
Point of care
Dry eye disease
Lateral flow assay
Interleukin 6
Lactoferrin
Molecular diagnosis

1. Introduction

Dry eye disease (DED) is the inflammation of ocular surface with symptoms of visual disturbance and tear film instability [1]. It is the most common eye disease with 16 million patients estimated in US and 128 million patients worldwide [2]. The global economic impact is more than $55 billion dollars per year. DED is more prevalent in the elderly and women [3]. In recent years, the widespread use of digital devices and contact lenses have significantly increased the prevalence of DED, particularly in the office workers and young adults [4]. It has been estimated that the prevalence of DED ranges from ~20 to 50% of the global population [3]. Thus, DED has become a major health challenge in our society with significant unmet medical needs. The National Health Institute has also recently designated July as “Dry Eye Awareness Month” [5].

Multiple factors contribute to the onset of DED, including aging, genetic predisposition, environmental factors and other preexisting diseases (such as refractive surgery, diabetes, etc.). DED is clinically subdivided into two subtypes: one with reduced tear secretion (aqueous-deficient DED, 10% of DED patients), and one with increased tear evaporation (hyper-evaporative DED, 80% of DED patients including mixed types). The resultant tear hyperosmolarity directly or indirectly initiates a vicious cycle of epithelial stress and desiccation, inflammatory events, and ocular surface damage [6]. DED patients commonly experience discomfort, pain and altered visual acuity. In the severe stage, DED impairs functional vision and prevents patients from carrying on daily activities such as reading, driving and computer related works with lowered productivity and significantly reduced quality of life.

Because DED is a multifactorial disease of the tear film with different underlying molecular mechanisms, it remains a challenge with its diagnosis and treatment [7]. The current diagnosis of DED is based on a combination of signs and symptoms, with conventional methods including tear break up time (TBUT), fluorescein staining, ocular surface disease index (OSDI) and Schirmer’s test [7]. All these methods require an expensive visit to an eye doctor’s office, which can significantly delay the diagnosis of DED. Most importantly, the interpretation of the results of these conventional tests depends on the subjective judgment of the examiner. Since early diagnosis is the key to prevent DED from progression, a POC, in-home diagnostic kit is essential to fill the gap and...
Some limitations of this microfluidic device were reported as 1) fluorescence emission was used for data reporting 2) lacking of the assay sensitivity for low lactoferrin concentration samples. Most importantly, clinical studies and a meta-analysis have shown that there is a significant decrease of lactoferrin protein levels observed in the tears of DED patients comparing to the controls [13,14]. Recently, encouraging results were reported that a novel microfluidic device could detect lactoferrin concentrations in tear samples with positive correlation with the severity of DED [15]. Some limitations of this microfluidic device were reported as 1) fluorescence emission was used for data reporting 2) lacking of the assay sensitivity for low lactoferrin concentration samples.

IL-6 is an active cytokine which can be transiently induced at the injured tissues or inflammation sites and is responsible for acute phase responses and immune reactions [16]. Clinical studies have shown that the levels of tear IL-6 correlates excellently with DED symptoms and progression [10,17]. A significant elevation of tear IL-6 has also been observed after 2 weeks of wearing soft contact lens [18]. Studies have shown that the risk of developing DED increases by 2 to 3-fold due to wearing contact lens [19]. It has been suggested that IL-6 may be an indicator of a worsening epithelial and mucoid function of the ocular surface [17]. In summary, both lactoferrin and IL-6 are validated molecular biomarkers and can be used for the development of early diagnosis methods of DED.

Lateral flow immunoassays (LFIA) are rapid tests that have revolutionized POC diagnosis and patients’ self-monitoring management of various diseases [20]. The success of LFIA has translated laboratory-based immunoassays into accessible home testing devices with advantages including great affordability, ease of use, a short turnaround time, and a high accuracy. Briefly, a testing sample is placed on a conjugation pad where the analyte (or antigen) of interest is bound by conjugated antibodies using gold nanoparticles (AuNP) as probes. The analyte-antibody mix subsequently migrates along a membrane by capillary action across both the “test line” and the “control line” on the lateral flow paper strips. These “lines” on the strips are coated with antibodies which can capture the analyte of interest in a sandwich fashion. A positive test is confirmed by the appearance of the AuNP colored test lines and control lines.

The goal of this study to develop a sensitive LFIA for POC diagnosis of DED with dual biomarkers (lactoferrin and IL-6) in the tear samples. The use of two (or more) biomarkers is to address the challenges of complex multifactorial causes of DED. Given the limited accuracy and poor clinical correlations of current diagnosis methods, targeting dual biomarkers for multiplexing detection should increase the accuracy of POC DED diagnosis. A standardized tear sample collection protocol was also developed with the use of disposed contact lenses (with patient’s consent), which is another crucial element for the success of DED diagnosis [21]. In addition, quantitative data from LFIA images were also analyzed for the accurate data reporting. The initial testing of 20 tear samples from confirmed DED patients and healthy individuals provides positive LFIA results for the large-scale clinical studies.

2. Materials and methods

2.1. Reagents

Human purified rIL-6 protein and lactoferrin (lyophilized powder) were purchased from Sino Biological (Wayne, PA) and Thermo Fisher Scientific (Waltham, MA), respectively. The purified rabbit IL-6 polyclonal antibody and lactoferrin polyclonal antibody were purchased from Thermo Fisher Scientific (Waltham, MA) and Sigma Aldrich (St. Louis, MO), respectively. The purified mouse IL-6 monoclonal antibody and mouse lactoferrin monoclonal antibody were purchased from Biolum (San Diego, CA) and Thermo Fisher Scientific (Waltham, MA), respectively. The laminate backing card, nitrocellulose membrane, sample/absorbent pads were purchased from Sigma Aldrich (St. Louis, MO). The goat anti-mouse antibody and ELISA detection kits for human IL-6 and lactoferrin were purchased from Thermo Fisher Scientific (Waltham, MA). The clear-bottom polystyrene 96 well plates were purchased from Corning Incorporate (Tewksbury, MA).

2.2. Assembly and production of lateral flow paper strips

Lateral flow paper strips were produced with the published procedures with minor modifications [22]: 1) overlapping sample pad, conjugated pad, nitrocellulose and absorbent pad were mounted onto a backing card as shown in Fig. 1A; 2) while the 1st antibody was conjugated to SPA AuNP on the conjugated pad, the 2nd antibody was printed onto the test line and the anti-mouse antibody was printed onto the control line of the assembled paper; 3) To avoid possible false negative results, an additional “hook line” was created for lactoferrin LFIA paper strips with the purified lactoferrin (Fig. 1B). The disappearance of Both hook line and test line will confirm that the highly concentrated lactoferrin in tear have saturated the binding capacity of AuNP labeled lactoferrin antibody with false negative results. 4) LFIA paper were cut into 5 mm strips for the use of LFIA tests.

2.3. Procedure of lateral flow assays

For testing with purified proteins, the serial diluted lactoferrin and rIL-6 solutions were made with a pH 7.4 buffer with protein concentrations indicated in Fig. 2. A volume of 50 μL of the protein solution at different concentrations was loaded onto the sample loading zone of the lateral flow paper strips. For tear sample testing, 1 drop of contact lens washed solution (≈50 μL) was loaded onto a LFIA testing strip. To avoid the false negative results of lactoferrin test, a 2x diluted tear sample (see details in the Tear sample collection section) of ≈50 μL (1 drop) was used for retesting when the hook line and test line were both absent in a LFIA test. Each sample was allowed to flow from the loading zone to absorbent pad on a LFIA testing strip by capillary action at the ambient temperature. After 10 min, visual results of LFIA paper strips were captured with a smartphone camera followed by ImageJ software analysis.

2.4. Enzyme linked immunosorbent assay (ELISA)

ELISA tests were performed according to the manufacturing
instructions with a Corning’s clear-bottom polystyrene 96 well plate. The purified lactoferrin and rIL-6 with serial diluted concentrations were added to the wells of each test plate. After BSA blocking and buffer washing steps, the HRP conjugated antibodies and TMB substrate were added to complete the ELISA reaction. After addition of the ELISA stop solution, the assay plates were measured for absorbance reading at wavelength of 450 nm with an Accuris MR9600 microplate reader (Edison, NJ). The ELISA results were averaged from triplicate testing sets after background absorbance subtraction.

2.5. Tear sample collection and storage

De-identified and discarded contact lenses were collected with the consents of the trial participants and were provided by Westview Eye Institute (San Diego, CA). All DED patients had confirmed diagnosis with chronic DED symptoms and met at least 3 of the following 4 dye eye

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Fig. 1. A) Schematic diagram of LFIA for detection of IL-6 with a test line and a control line; B) Schematic diagram of LFIA for lactoferrin testing with an additional hook line (circled) between the test line and control line. The tear sample flows through the LFIA paper strip by capillary action while the analyte (IL-6 or lactoferrin) binds to the antibody at the test line.

Fig. 2. Determination of the lowest detection limits of LFIA for A) IL-6; B) lactoferrin using serial diluted purified proteins. The red arrows indicate the lowest detection concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
lenses were carefully washed with 50 μL of 50 mM Tris buffer (pH7.4) with 10% glycerol. The obtained 20 tear samples (10 in each group) were labeled and tested immediately or stored at 4 °C for later testing. For long-term storage, the tear samples were stored at −20 °C without frequent freezing and thawing.

In home testing, one drop of buffer (estimated volume of ~50 μL) or filtered home-used water can be used as no significant differences of LFIA data were observed between filtered home-used water vs pH7.4 buffer. When the hook effect was observed with LFIA results (Both test line and control line were negative) from lactoferrin tests, two drops (~100 μL) of buffer or filtered water should be used to wash the contact lens, while still loading ~50 μL for LFIA retesting. This can create a 2x diluted tear sample to avoid possible false negative results for the highly concentrated lactoferrin tear samples.

2.6. ImageJ data analysis

A smartphone (iPhone 11 pro, Apple, Cupertino, CA) was used to record images and videos of LFIA testing strips. The smartphone was attached to the frame of a desktop stand for support during the dynamic measurements (locked exposure, fixed focus, and controlled illumination). ImageJ version 1.53k (open source software from NIH, Bethesda, MD) was used for image calculation to 16-bit, followed by a plot profiling of the selected LFIA strip. Gray scale values of each test line and control line were calculated by subtraction of background signals from original gray scale value.

2.7. Statistical analysis

The student t-test was conducted using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). The statistical significance was determined by P-values as follows: P-values of <0.05 (*), <0.01 (**), and <0.001 (***)). The mean ± standard deviation (SD) was obtained from at least three separate experiments.

3. Results

3.1. Establishment of LFIA for IL-6 and lactoferrin detection with purified proteins

In order to determine the minimal detection limit of LFIA, purified lactoferrin and recombinant IL-6 were used for LFIA assay development and evaluation. As shown in Fig. 2A, a visible testing line can be observed starting at 0.1 ng/mL of IL-6, with strong signals recorded at 1, 10, and 100 ng/mL, respectively. At 1000 ng/mL, a negative test line was observed due to the highly concentrated and unlabeled IL-6 binding to the 2nd IL-6 antibody, while slowly migrating AuNP conjugated 1st antibody couldn’t be captured at the test line. Since normal tear IL-6 level is well below 1000 ng/mL, the current detection range from 0.1 to 100 ng/mL is well suited for POC tests for DED diagnosis at home.

Lactoferrin is an abundant tear protein with normal tear concentration of 2.2 mg/mL. Highly concentrated lactoferrin in tears can saturate all available AuNP labeled antibody at the loading zone. The unbound lactoferrin molecules can travel faster than the AuNP-antibody bound-lactoferrin complex and occupy the available 2nd antibody binding sites at the test line. Thus, no more binding capacity is available to the colored AuNP-1st antibody-lactoferrin complex at the test line. As a result, no visible test line is observed with reporting of a false negative data. This phenomenon is called “Hook effect” or “Prozone phenomenon” [24]. We designed an additional hook line with purified lactoferrin printed onto the lateral flow paper strip between test line and control line (Fig. 1B). Normally, unbound AuNP-1st antibody can bind to lactoferrin at the hook line with a visible line. When tear lactoferrin is overloaded, no more unbound AuNP-1st antibody is available. Thus, both testing line and hook line are negative while the control line is positive. This is when false negative results are reported. In this case, the protein or tear samples are required to be further diluted for retesting. As shown in Fig. 2B, the minimal detection concentration of purified lactoferrin was estimated at 10 ng/mL for the lateral flow assay.

3.2. Confirmation of LFIA results using conventional ELISA

In order to further confirm the results from LFIA testing, the conventional ELISA assay was performed with serial diluted IL-6 and lactoferrin. ELISA results demonstrate the minimal detection limits for IL-6 and lactoferrin to be around 0.08 ng/mL and 6.25 ng/mL, respectively (see results in the Supplemental Fig. S1). Since the ELISA assay has a wash step to remove nonspecific protein binding, it was expected that ELISA assay is more sensitive than LFIA. In summary, our ELISA results further validated the data from LFIA testing which made it possible for the POC testing of IL-6 and lactoferrin proteins in human tear samples.

3.3. Testing IL-6 and lactoferrin of tear samples from DED & controls with LFIA

The 20 archived tear samples (10 each group) from DED and healthy individuals were applied to the lateral flow paper strips. All 10 DED samples showed positive test lines of LFIA testing on IL-6 (Table 1). The 20 archived tear samples (10 each group) from DED and healthy individuals were applied to the lateral flow paper strips. All 10 DED samples showed positive test lines of LFIA testing on IL-6 (Table 1). As shown in Fig. 3 (left), a visible testing line can be observed starting at 0.1 ng/mL of IL-6, with strong signals recorded at 1, 10, and 100 ng/mL, respectively. At 1000 ng/mL, a negative test line was observed due to the highly concentrated and unlabeled IL-6 binding to the 2nd IL-6 antibody, while slowly migrating AuNP conjugated 1st antibody couldn’t be captured at the test line. Since normal tear IL-6 level is well below 1000 ng/mL, the current detection range from 0.1 to 100 ng/mL is well suited for POC tests for DED diagnosis at home.

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3.4. Quantitative image data analysis

Efforts have been made to quantify the digital density of the test lines with the ImageJ software. After converting the raw lateral flow images into 16-bit gray scale format, gray scale values at the test line were recorded by the subtraction of the background signals. Significant differences between DED patients and normal controls were observed with P < 0.001 for both IL-6 and lactoferrin tests. Fig. 3A exhibited the gray scale results of 10 tear samples for IL-6. The average gray scale value at test lines for IL-6 of DED patients was 37.7 ± 2.8 compared to that of normal controls (9.1 ± 0.9). The 41-fold difference in gray scale value for DED test line vs normal test line gave a broad dynamic range for accurate data quantification of IL-6 tests. On the other hand, the average gray scale data at the test lines for lactoferrin of normal controls was 19.8 ± 3.4 while being 1.7 ± 3.5 for DED patients. The dynamic range was 11-fold difference in gray scale value. In addition to a relatively lower gray scale value at the lactoferrin test line, there was one outlier in DED tear sample with high gray scale value of 12 (D8, Fig. 4B). Although the gray scale value decreased 40% compared to that of the average of normal control, this LFIA test still showed positive results (Table 1). Thus, the quantitative analysis of lactoferrin tests by ImageJ are essential to avoid inaccurate data interpretation.
Fig. 3. Representative images of LFIA tests with DED and control tear samples for IL-6 (left) and lactoferrin (right). The red arrow indicates the results on the testing line of LFIA tests. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 4. Quantitative analysis with ImageJ software of LFIA data from 10 DED samples (D1 to D10) and 10 normal control (N1 to N10) tear samples for testing on A) IL-6 and B) lactoferrin. The insets show the results of statistical analysis with determination of P value.
4. Discussion

The early diagnosis of DED has posed a significant challenge as various factors are involved in the process of DED pathogenesis [1]. Current diagnosis mainly relies on the visitation of an eye doctors’ office with the aforementioned tests. However, these subjective and objective clinical observations do not always correlate [25,26]. Some patients in considerable discomfort have no significant clinical signs, while those with severe dry eye and vision-threatening ocular complications suffer from only mild symptoms [25]. In addition, the subjective symptoms of DED are often nonspecific due to the multifactorial nature of DED. The most effective ways to address these challenges are 1) Identification of relevant biomarkers exhibiting strong correlations with DED symptoms and progression; 2) Establishment of POC diagnostic methods with these biomarkers; 3) Multiplexing POC tests at the early stage. POC diagnosis is well suited for the early diagnosis of DED. The lateral flow test offers a convenient in-home testing platform which has already been successful in the early diagnosis of pregnancy [20]. Recently, a lateral flow rapid test of MMP-9 in the tear film has become clinically available [27]. Clinical studies showed encouraged data with MMP-9 positive results identified in 19/47 or 40.4% of DED patients. Positive MMP-9 results also correlated well with subjective symptoms of DED evaluated by OSDI, TRUS and Schirmer test results [28]. Although many limitations remain, this MMP-9 LFIA test points a positive direction for the future diagnosis of DED.

Our LFIA test platform (see the graphic image) on IL-6 and lactoferrin are a step closer to achieving multiplexing tests for the in-home diagnosis of DED. Since IL-6 appears at the onset of inflammatory processes, it is expected to be a more reliable early diagnostic indicator comparing to MMP-9. This ubiquitous matrix protease is usually expressed after the IL-6 amplifier (IL-6 Amp) is triggered and diseased cells begin invading neighboring tissues [29]. The fact that all 10 tear samples exhibit significantly elevated levels of IL-6 with a 41-fold dynamic range demonstrated a strong correlation of tear IL-6 levels with DED clinical symptoms. In the future, kinetic LFIA testing of tear IL-6 from DED patients can provide significant clinical values in monitoring DED progression and treatment effects.

Lactoferrin is a major defensive component in the tear film and prevents bacteria and viruses from growing on the ocular surface. Many reports have demonstrated that the decrease of lactoferrin accompanies the course of DED progression [11,13,14]. 9 of 10 DED tears in our lateral flow tests show negative results, suggesting a reverse correlation of tear lactoferrin levels with DED symptoms. In order to determine the one positive case (DED sample D8 in Fig. 4B) of DED tear samples, a quantitative analysis was performed at the test line of the lateral flow images. This DED tear sample showed reduced lactoferrin levels at around 1 mg/ml, but this concentration was still above the detection limit of our lateral flow assay. Previously, researchers have suggested a cut-off value of 1.1 mg/ml for tear lactoferrin so that the assay has optimal accuracy for the diagnosis of DED [30]. Thus, a quantitative data analysis is beneficial to accurately report LFIA data, particularly for tear samples with high expressed protein levels such as lactoferrin. In addition, smartphones as LFIA readers have been transformed into a universal integrated platform for imaging, data processing, and storage, thus providing quantitative results in low-resource settings [31].

Although none of our 10 normal tear samples showed false negative results due to the “hook effect” from highly concentrated tear lactoferrin, an additional step line is necessary to eliminate inaccurate results in lactoferrin LFIA tests. Recently, Ross and colleagues published a comprehensive study on high antigen concentration effects in sandwich LFIA [32], their data showed that digitally analyzing the LFIA data allows clear differentiation of highly positive samples and false negative samples. It can be also determined whether the LFIA is within the dynamic working range or at critically high concentrations. Since hook effect could play a significant role in the accuracy of LFIA, further investigations with more LFIA data are crucial before the implementation of in-home LFIA testing.

Tear sampling provides a convenient, non-invasive method for analyzing biomarkers in preventive and personalized medicine. However, obtaining reproducible and unaltered samples is challenging because of the small sample volumes of tears [21]. LFIA platform for DED diagnosis utilizes standardized protocols for tear sampling with disposable contact lens from tested individuals. Although the test participants need to wear contact lens for 1–3 h, this tear collection method has eliminated inaccurate tear dilution or non-uniformed elution of tear proteins. Future applications will allow us to further evaluate this novel method and its impacts on the quality of LFIA results.

Although encouraged results were obtained from this initial small-scale study of 20 tear samples, cautions need to be taken on the LFIA data of IL-6 and lactoferrin from in-home tear tests. For example, some systematic diseases (including flu infection, rheumatic arthritis, HIV, etc.) can induce high levels of IL-6. The comprehensive medical history of a particular patient is necessary for accurate interpretation of elevated IL-6 results in tear samples. On the other hand, negative results of lactoferrin LFIA tests need to be confirmed by diluted tear samples, so false negative data of lactoferrin can be excluded from further consideration. Thus, more clinical investigations with larger tear sample sets are warranted to further confirm this dual biomarker DED diagnostic method. For the success of future clinical studies, the selection of trial participants with a well-defined etiology is the key to validate this POC method. Lastly, this paper based lateral flow assay can be manufactured at a large-scale with relatively low cost (below ~$1 per test), which can be widely affordable to the global DED patients both in the developed and developing countries.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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