Original Article

# The Ability of Antibody against Truncated Matrix Metalloproteinase 9 Peptide to Evaluate the Native Protein on Tear Drops of Dry Eye Disease Patients by a Point-of-Care Diagnostic System

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## Abstract

**Purpose:** To obtain a reactive and specific antibody against truncated matrix metalloproteinase 9 (MMP-9), that has reactivity toward the native protein. Precision, accuracy, specificity, and sensitivity were evaluated using a point-of-care test.

**Methods:** An *in silico* study was used to confirm the anti peptide truncated MMP-9 is against native MMP-9. After an antibody titer assessment, purification, and characterization, the anti MMP-9 was assessed. The cut-off value was determined using the purified gelatinases of the supernatant HCT 116 cell line. The supernatant was purified by preparative native-polyacrylamide gel electrophoresis based on charge and size of the proteins. Furthermore, quality control (QC) of the results were performed following standard densitometry methods.

**Results:** A truncated MMP-9 is the major epitope peptide that can trigger the immune system to scavenge for a specific and reactive antibody against the native MMP-9. The MMP-9 native protein is purified from the supernatant of the HCT 116 cell line and the commercially available, full-length MMP-9. The cut-off value was estimated at 30 µg/mL. QC results indicated that the specificity was 80%, sensitivity was 96.7%, accuracy was 91%, and precision was 91.66%. The area under curve was 0.827 ($P < 0.001$). The positive predictive value was 83%, and the negative predictive value was 96%.

**Conclusions:** The antibody against the synthetic epitope peptide can detect the native MMP-9. Native MMP-9 is considered the main biomarker in an immunoassay POCT and is used to diagnose dry eye disease (DED). In accordance with QC results, MMP-9 point of care test can be utilized for screening patients suffering from DED.

**Keywords:** Dry eye disease, Epitope peptide, *In silico* analysis, Matrix metalloproteinase 9 point-of-care test, Polyclonal antibody

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## Introduction

Dry eye disease (DED) has a 20% to 50% worldwide prevalence and is considered a serious health concern.[1] A DED diagnosis requires a tear film analysis that looks at the changes in three components: mucin, lipids, and the aqueous layer. All three of these can impact the stability of the tear film.[2]

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Oxidative stress can increase inflammatory mediators such as cytokines and matrix metalloproteinases (MMPs); these changes can lead to tear film instability.[2] MMPs (such as gelatinizes) can result in extracellular matrix degradation, which result in the pain experienced by DED patients.[3] Pro-MMP is a zinc and calcium-dependent protease that contains 4 components: a propeptide truncated domain, a fibronectin domain, a catalytic domain (Zn2+ binding domain), and a hemopexin domain.[4] Gelatinases (MMP-9/-2) have 3 consecutive fibronectin domains. MMP-2 is a regulatory protease that can activate MMP-9, which is a functional protease.[5]

High levels of MMP-9 are considered important biomarkers for diagnosing, classifying, and monitoring DED.[6] Experimentally, the use of antibodies in therapy is due to the ability of their epitope to detect a cellular and humoral immune response. An *in silico* analysis can estimate how accurately the epitope can detect this response.[7]

Experimental epitope mapping is an expensive, laborious, and time-consuming component of accurate epitope detection.

There are several databases that can predict different epitope harboring properties such as immunogenicity, solubility, and stability. Through *in silico* studies, we confirmed that certain epitopes can trigger the immune system to generate reactive antibodies against the native MMP-9 that is expressed on the cell surface.

The polyclonal antibody produced for this experiment is used to evaluate the MMP-9 point-of-care test (POCT) in six several areas such as accuracy, precision, sensitivity, specificity, positive predictive value, and negative predictive value. These were all examined in the tear samples of patients with DED.

## Methods

An *in silico* study is the best tool to design and evaluate an epitope sequence. Despite being time-consuming and expensive, the *in silico* study is the best tool to design and evaluate an epitope sequence. The protein sequence (FASTA) of the MMP-9 protease was obtained using UniProt Knowledgebase at http://www.uniprot.org. The MMP-9 sequence was used for a BLAST search at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Blast proteins are used to find similar sequences among all living beings. The most accurate region of the B-cell epitope containing MMP-9 was determined by the immune epitope database (IEDB) server.[8] The Bepipred tool and BepiPred-2.0 were used to predict the linear B-cell epitopes of the selected peptide sequence peptide at http://tools.iedb.org/bcell/.[9]

Expasy's ProtParam (http://us.expasy.org/tools/protparam.html)[10] was used to conduct a physicochemical parameters evaluation. The theoretical isoelectric point, the total number of positive and negative residues, molecular weight, and grand average hydropathy, were calculated.

All animal experiments were performed in accordance with the approved guidelines of the Ethics Committee of Tarbiat Modares University (approval ID number: IR.REC.1397.084). To prepare the antibodies, the 58 amino acid (aa) peptide sequence 232–289 (synthesized by SYN high quality peptide company, China) was utilized and injected into female New Zealand white rabbits. These rabbits were 8 weeks old at the time of the initial injection. These rabbits were injected every 2 weeks for a total of 8 weeks. The complete adjuvant was used for the initial injection as the main injection. Also, the Freund's incomplete adjuvant was used labeled as the booster injection and was used for the second, third, and fourth injection. A blood sample was collected after the fourth injection, and using a prepared serum, an indirect enzyme-linked immunosorbent assay (ELISA) test was performed.

One milliliter of phosphate-buffered saline (PBS) in a stable emulsion (containing 500 ug of synthetic epitopic MMP-9 peptide, and an equal volume of Freund's complete adjuvant) was injected into the female white rabbits.

Every 15 days, booster injections were administered. At this interval, 250 ug/500 uL of the peptide was mixed with Freund's incomplete adjuvant.[11]

After the fourth injection, a blood sample was collected, and an ELISA test was performed to measure the antibody titer against 1 ug/well of the synthetic peptide. One hundred microliter of PBS was applied on to the wells of the microtiter plate and incubated at 37°C overnight. One ug/well of bovine serum albumin (BSA) in 100 uL of PBS was coated on to the wells as the nonspecific binding agent.

Next, 5% skim milk was used in the wells for 1 h at 37°C. Then, the wells were blocked and incubated with 100 uL/well of serial diluted serum (1:500 etc.), for 1 h at 37°C. These wells were also incubated with 100 uL of 1:4000 diluted horseradish peroxidase-conjugated mouse antirabbit immunoglobulin G (IgG) (Thermo Scientific, MA, USA) for 1 h at 37°C. After each phase, the wells were removed for three times. One hundred microliter of tetramethylbenzidine substrate reagent (BD Biosciences Pharmingen, CA, USA) was added and incubated for 10 min at 37°C. Then 50 μL of 1 M HCl was added and the absorbance was measured at 450 nm.

The serum collected from the ear vessels of the rabbits was precipitated with a 50% ammonium sulfate saturation. Precipitated proteins were dissolved in PBS and dialyzed against 10 mmol of PBS at a pH of 7.2. They were then transferred into a protein-A affinity chromatography column according to the manufacturer's guidelines. IgG's binding was eluted in 0.1 M citric acid at a pH of 3.0 and then collected in three separate vials. These samples were immediately neutralized to physiological pH by adding 500 μL in 1 M of tris-base buffer at a pH of 9. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the standard procedure. The indirect ELISA demonstrated that the purified polyclonal antibody could react toward both the native MMP-9 protein (purchased from Boster company)

and the gelatinase secretory proteins (72 [e.g., MMP-2]/82 kD [e.g., MMP-9]). This was shown in the supernatant media of the HCT-116 colon cancer cell line that was prepared and purified by preparative native PAGE.

Native gel electrophoresis in the absence of SDS was performed and proteins were eluted and homogenized by an elution buffer containing 50 mM of tris-HCl, 0.1 mM of ethylenediaminetetraacetic acid, and 15 mM of NaCl. Each band was added to a 50 kD amicon column for further purification and concentration of the solution. The inhibition ELISA was performed using the referenced coated peptide. A 20 aa peptide sequence (FPFIFQGQSYSACTTDGRSD) was used to inhibit the reaction between the antibody and the coated peptide. This peptide sequence was selected because it contains the epitope from the MMP-9 proteins. Furthermore, a 12 aa peptide sequence (*MHPNAGHGSLMR*) was also utilized. Shoari *et al*. reported that this 12 aa peptide sequence inhibited MMP-9 during a phage display peptide experiment.[12]

Prospectively, MMP-9 POCT was performed on DED patients with the following four criteria:

- Patients who chose to participate voluntarily, did not use corticosteroid medications and antibiotics, were not pregnant,[13] and consented to not washing their face and eyes 1 h before their tear samples were collected[14]
- An ophthalmologist evaluated all of the patients and analyzed their tear film break-up time (TFBUT). Upon assessment, key symptoms reported by patients included ocular fatigue, dryness, and itching
- A tear sample was obtained by squeezing 100 uL of artificial tear solution in the patient's eye and then it was collected by an applicator tool and stored in a 0.2 microtube at −20°C
- The consent form was not required, because tear sampling was a noninvasive and easy procedure.

The POCT strip contained three pads: a conjugate pad, a sample pad, and an absorption pad. The strip also had a nitrocellulose membrane and a backing card. Gold nanoparticles conjugated with anti-MMP-9 were sprayed on the conjugate pad. The conjugated antibody can react to protein-A that has been sprayed on the control line region. The polyclonal antibody sprayed on the nitrocellulose membrane (test line region) can detect tears containing MMP-9. The sample and conjugated pads were treated with buffers. The buffer used to treat the sample pad contained 0.25 g of sucrose, 0.5% of tween 20, 5 mL of PBS, and 0.025 g of BSA. The conjugate pad was treated with a buffer that contains 0.25 g sucrose, 0.5% tween 20, 5 mL PBS, and 0.05 g BSA. A 50 µL tear sample was sprayed on the sample pad and it was left to dry for 1 h at 37°C.

Cut-off values were measured using a purified supernatant HCT-116 cell line gelatinase by native-PAGE based on the charge and the size of the proteins.

The receiver operating characteristic curve was used for the evaluation of sensitivity, specificity, and the area under curve (AUC) POCT. Accuracy, precision, positive predictive value, and negative predictive value were also evaluated using the formula.

- Accuracy: True positive + true negative/total population
- Precision: True positive/true positive + false positive
- Sensitivity: Number of true positives/number of true positives + number of false negatives
- Specificity: Number of true negatives/number of true negatives + number of false positives
- Positive predictive value: Number of true positives/number of true positives + number of false positives
- Negative predictive value: Number of true negatives/number of true negatives + number of false negatives.

## Results

*In silico* studies indicated that the experimental epitope index "FPFIFEGRSY" exists in a 58 aa peptide sequence (232–289) and is located on the MMP-9 fibronectin domain as *"FPFIFEGRSYSACTTDGRSDGLPWCSTTANYD TDDRFGFCPSERLYTQDGNADGKPCQ"*. The IEDB database was used to identify the most effective region of the epitope of the MMP-9 protein that can stimulate B-cells. The hydrophilicity, flexibility, surface accessibility, antigenicity, and turns were all considered as factors.

The results of the indirect ELISA demonstrated that the rabbits were immunized and triggered to create antibodies against the synthetic peptide. The titration curve indicated that the antibody [Figure 1] reacted toward the synthetic peptide, native commercial MMP-9, and gelatinases purified from the supernatant of HCT-116, even at a dilution factor of 1:32000.

Protein-A column chromatography gathered three separate samples the purified antibodies. These were identified via SDS-page gel electrophoresis [Figure 2]. Fraction 2 was utilized to assess cross reactivity in the ELISA analysis [Figure 3]. Native PAGE confirmed that two bands (72/82 kD gelatinases) of the eluted proteins of the HCT 116 cell line supernatant were in lane 2.

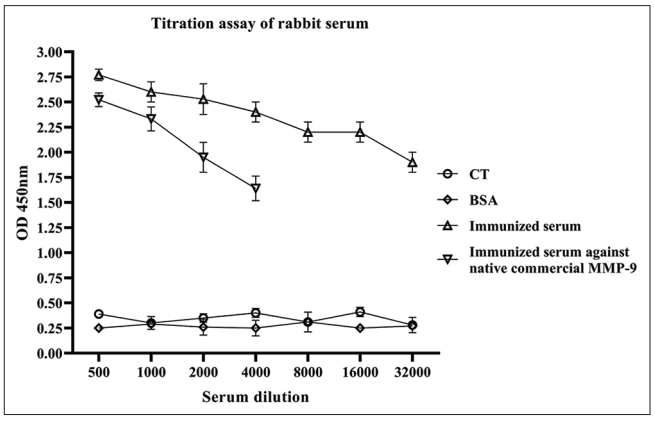


**Figure 1:** Titration curve of normal rabbit and immunized rabbit serum. BSA: Bovine serum albumin, CT: Control serum (nonimmunized), MMP-9: Matrix metalloproteinase 9

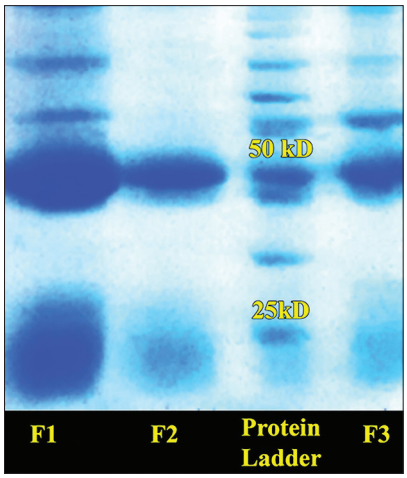


**Figure 2:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed two bands 50 kD H-chains and 25 kD L-chains related to immunoglobulin G (s) in fraction 2 (F2). Lane 1, 2, 4: Fraction 1, 2, and 3 of purified pAb. F2 was selected for further experiments

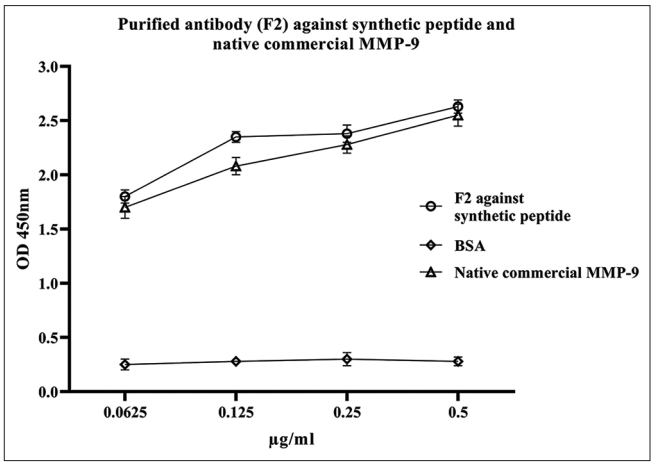


**Figure 3:** Reaction of immunoglobulin G purified antibody fraction 2 with synthetic peptide, bovine serum albumin, native commercial matrix metalloproteinase 9, OD value of control serum (1/500) about 0.32. MMP-9: Matrix metalloproteinase 9, BSA: Bovine serum albumin

The reaction of the purified antibody (F2) against the lane 2 eluted proteins was determined by ELISA and Western blotting [Figure 4]. The inhibition immunoassay or inhibition ELISA was performed by measuring the antigen concentration by signal detection [Figure 5].

Patients with clinical symptoms of chronic irritation, fatigue, redness, itchiness, and dryness were considered for DED evaluation. The Schirmer test and the TFBUT were performed on all of the patients. For our study, only those patients with a Schirmer test score <10 mm and a TFBUT of <10 s were included in our analysis.

The POCT band was divided into two groups: positive and negative. In the MMP-9 POCT, 30 patients were considered as positive, and 24 of the 30 patients were considered negative. There were no false negative patients in this cohort. Thirty patients were regarded as positive, without the presence of any false negative patients within the group and 24 out of the 30 patients were negative. The cut-off value for the MMP-9 POCT was estimated to be around 30 ug/mL.

According to formula, the diagnostic abilities were evaluated as 80% specificity, 96.7% sensitivity, 91% accuracy, and 91.66% precision. The AUC was 0.827 ($P < 0.001$) [Figure 6]. The positive predictive value was 83% and the negative predictive value was 96%.

## Discussion

Biological biomarkers such as MMP-9 can be utilized to accurately diagnose and monitor DED patients.[15] B-cell epitope mapping was used to select the ideal MMP-9 epitope peptide sequence for preparing the appropriate antibodies that are both reactive and specific.[7] The propensity of aas is most important for prediction of linear epitopes on B-lymphocyte cells. This property is in association with variety of agents. The selected important parameters are accessibility, hydrophilicity, flexibility, exposed surface, and polarity turns.[16] Gelatinases have a fibronectin domain that contains catalytic sites in MMPs. They are responsible for cleaving proteins that maintain the barrier and consequently they disrupt the corneal epithelial layer.

Gelatinases have a fibronectin domain that contains catalytic sites in MMPs. They are responsible for cleaving proteins that maintain the barrier and consequently they disrupt the corneal epithelial layer.

The activation of proMMP-9 was regulated by gelatinase A/MMP-2 protease. According to the IEDB database (September 2022), the 10 aa peptide sequence in the fibronectin domain of the gelatinase "FPFIFEGRSY" is the main epitope that can stimulate the immune system. Trolle *et al*. demonstrated that smaller peptide sequences with 9–10 aa are usually the ideal peptide size that MHC molecules utilize for T cell presentation.[17]

Furthermore, they found that three sequences that start with I, K, or F have the strongest binding affinity to the human leukocyte antigen allele. Ultimately, the importance of epitope 232–289 peptide sequence was confirmed in IEDB database. This sequence contains the experimentally verified "FPFIFEGRSY" epitope which starts with the F (Phe) residue. However, in a similar *in silico* study, epitope "FPFIFQGQSY" was verified as the linear epitope. The property of this epitope is due to the highly hydrophilic "R" aa in its sequence, which can increase the exposure of the hydrophilic surface and stimulate the immune response. The property of this epitope is due to the highly hydrophilic "R" aa in its sequence, which can increase the exposure of the hydrophilic surface and stimulate the immune response. This aa could increase the exposure of the hydrophilic surface and stimulate the immune system.

Furthermore, the inclusion of a hydrophilic "Q" aa in the sequence of "FPFIFQGQSY" epitope could increase the exposure of the hydrophilic surface and stimulate the

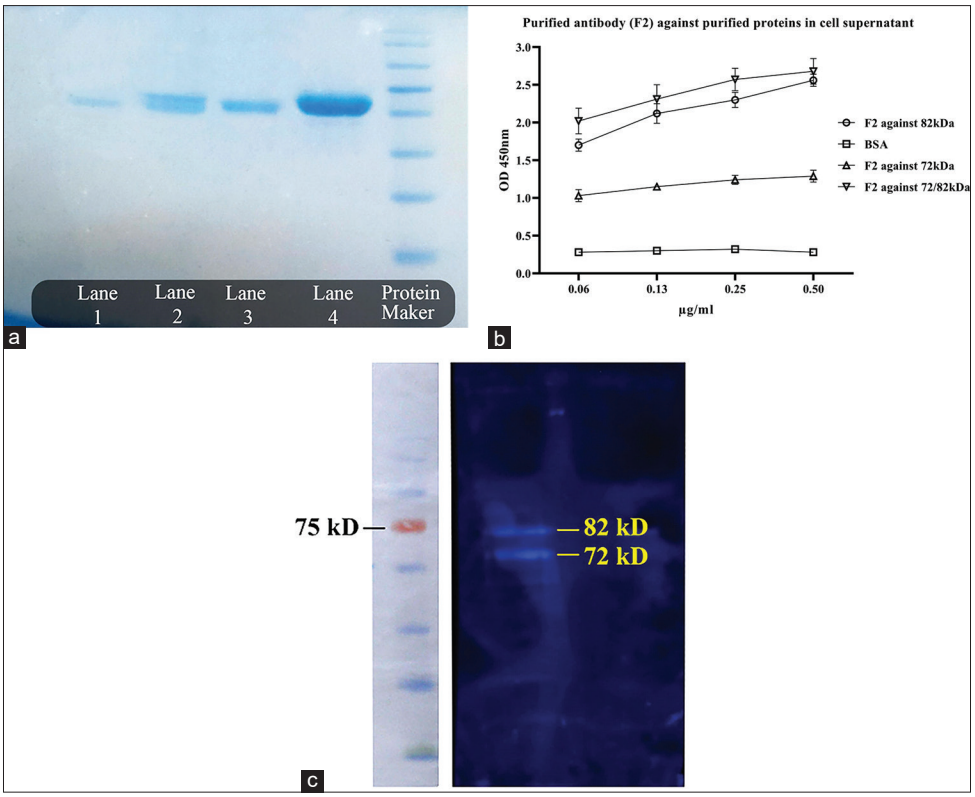


**Figure 4:** (a) Native polyacrylamide gel electrophoresis was performed on HCT 116 supernatant. 72/82 kD extracted proteins are located in lane 2. (b) Titration curve of purified immunoglobulin G fraction 2 against 82 kD matrix metalloproteinase 9 (MMP-9), 72 kD MMP-2 and a mixture of 72/82 kD proteins. (c) Western blot analysis of purified immunoglobulin G against gelatinases (from HCT 116 cell supernatant). BSA: Bovine serum albumin

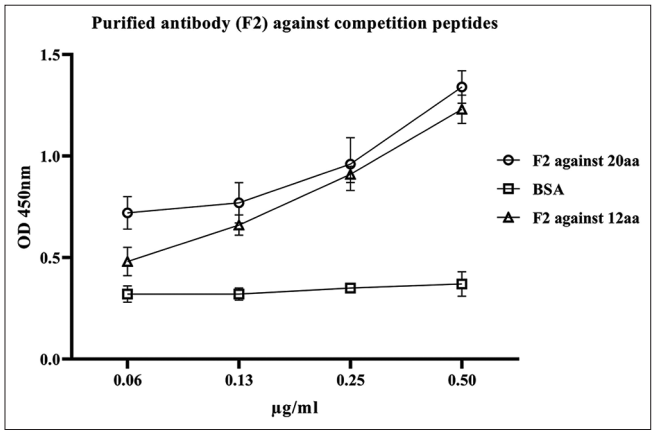


**Figure 5:** Titration curve of mixture of purified immunoglobulin G fraction 2 and inhibition peptides (20aa, *FPFIFQGQSYSACTTDGRSD*; 12aa, *MHPN AGHGSLMR*) against synthetic peptide. All performed in triplicates. BSA: Bovine serum albumin

immune system. Our findings confirm that the 10 aa sequence "FPFIFEGRSY" stimulates the animal immune system as the epitope index starts with F.[17] Contrary to previous findings, antibodies were produced against the predicted epitope sequence which consisted of a 58 aa synthesized peptide that can identify the native target protein (MMP-9) with a higher accuracy.[7] Increased levels of MMP-9/2 are a consistent finding in DED.[18] According to NCBI blast data, the gelatinases (MMP-9 and MMP-2) have a 58% identity in their sequences. Experimentally for evaluation of this identity, an ELISA test was performed and cross-reactivity antibodies of the serum rabbits were assessed.

Our experiment demonstrated that the epitope sequence in 58 aa synthetic peptides can stimulate the immune system and increase antibody levels. This antibody can detect MMP-2 with a low affinity on an indirect ELISA test. The MMP-2 was obtained by preparative SDS-PAGE.

The separation of gelatinases can be explained by the travel of charged molecules through a gel matrix when an electrical current is applied. The result of performed native PAGE 12% gel (based on mass or structure) in HCT 116 colon cancer cell line showed that two gelatinases "72 kD (e.g., MMP-2) and 82 kD (e.g., MMP-9)" were assessed in ELISA and a Western blot analysis. Western blot analysis confirmed that the 82 kD proteins (e.g., MMP-9) in the HCT 116 supernatant colon cancer cell line were detected by prepared and purified antibody (F2) against the synthesized epitope peptide.

The inhibition ELISA was conducted to measure the interaction between the inhibiting antigen and the antibody. The amount of free antibodies that are available to bind the coated reference antigen is directly dependent on the proportion of inhibiting antigen and antibody concentrations in solution. The inhibition ELISA demonstrated that the antibody against 58 aa containing the epitope index "*FPFIFEGRSY"* can inhibit synthesized peptide "*FPFIFQGQSY*SACTTDGRSD" that consists of

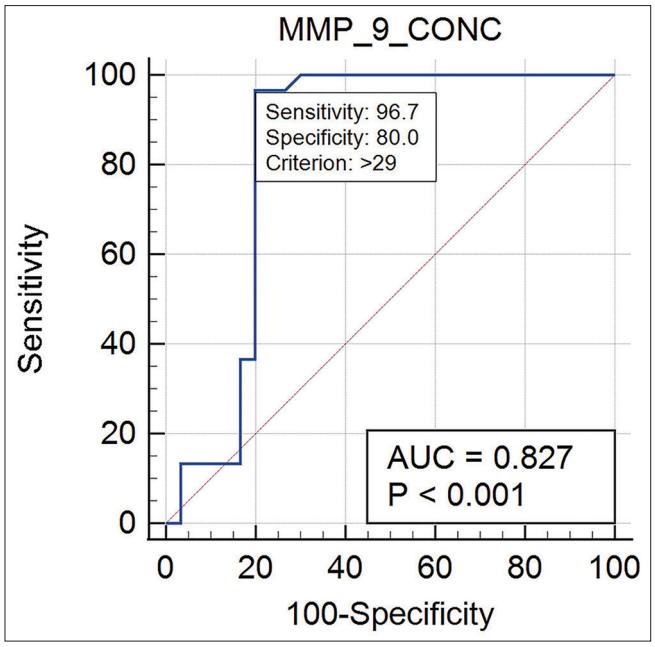


**Figure 6:** The diagnostic abilities were evaluated by receiver operating characteristic curve as 96.7% sensitivity, 80% specificity and area under curve with $P < 0.001$. MMP-9: Matrix metalloproteinase 9. AUC: Area under curve

the epitope index "FPFIFQGQSY." Furthermore, it was confirmed that the antibody can detect the sequence peptide "*MHPNAGHGSLMR*" a novel MMP-9 inhibitor. This sequence was acquired with using phage display performed by another study group in an indirect ELISA.[19]

Regarding Asia Dry Eye Society recommendation, there are two mandatory criteria for a DED diagnosis: clinical symptoms and TFBUT. A short TFBUT can cause visual failure and tear instability in DED patients.[2] Most studies demonstrate that MMP-9 activity has an important role in DED prevalence. Inhibiting MMP-9 activity as an important biochemical factor is considered. It was implied that MMP-9 is introduced for diagnosis and follow-up of DED. However, generally, it was considered a follow-up treatment option for DED patients.[14]

InflammaDry is the first MMP-9 PCOT that has been considered for tear film MMP-9 diagnosis in DED patients. It has an 85% sensitivity and a 94% specificity. MMP-9 inhibitors include corticosteroids, cyclosporine, tetracycline, and azithromycin. All of these can decrease MMP-9 activity.[14]

Sambursky *et al*. studied 237 patients that were evaluated due to a reduced fluorescein TFBUT, a reduced Schemer test or corneal fluorescein staining. These patients were detected by the InflammaDry test with 98% sensitivity and 81% specificity.[20]

Messmer *et al*. showed that in 101 patients, 47 DED patients and 54 control patients were diagnosed with DED due to an ocular surface disease index >12, a TFBUT <10 s, or a Schemer test <10 mm/5 min. When using the InflammaDry test on this cohort, 3 of the 54 control patients (5.6%), and 19 of the 47 DE patients (40.4%) were diagnosed with DED. Therefore, MMP-9 measurements in DE patients can be utilized as a new inflammatory biomarker for diagnosis and as an anti-inflammatory therapy target for DED.[21]

Our results show that MMP-POCT demonstrated clinically diagnostic ability. It can detect tear film MMP-9 in 29 out of 30 patients with a 96.7% sensitivity. Six of 30 control patients were diagnosed as patients after about 25 min. MMP-9 POCT with 80% specificity means that 20% of control patients were misdiagnosed to the patients. However, all of patients in patient group were diagnosed, correctly. Our data suggest that the POCT can be used as a primary diagnostic tool for DED patients with minimal concern for false negatives and a high specificity and a high sensitivity.

Furthermore, we found that the MMP-9 POCT can detect DED patients induced by systemic diseases as well. These are including cardiovascular disease, Sjogren's syndrome, diabetes, lupus erythematous, and rheumatoid arthritis. It was showed for the first by Dr. Henrik Sjögren in 1993 that infiltration of lymphocyte into the lacrimal and salivary glands in immune disease lead to DED symptoms.[22]

In conclusion, our study investigates if antitruncated MMP-9 against a 58 aa epitope peptide sequence "F P F I F E G R S Y S A C T T D G R S D G L P W C S T T A NYDTDDRFGFCPSERLYTQDGNADGKPCQ" can react with the native MMP-9. Overall, this can be utilized in the development of immunoassays (POCT) that assist in screening and diagnosing DED and detecting systemic diseases in DED patients.

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### *Conflicts of interest*
There are no conflicts of interest.

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