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BRIEF ARTICLE

MMP-8 analysis in gingival crevicular fluid using ELISA and novel chair-side test

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Abstract

AIM: To validate accuracy of a novel chair-side test for matrix metalloproteinase (MMP)-8 as compared to enzyme-linked immunosorbent assay (ELISA) in Periodontal health and disease.

METHODS: Gingival crevicular fluid was collected from 150 subjects, Group 1 (healthy) - 50 subjects, Group 2 (gingivitis) - 50 subjects and Group 3 (chronic periodontitis) - 50 subjects. A chair-side test strip was indigenously prepared using polyclonal antibodies (principle of immunochromatography) to detect the MMP-8 levels. The detection accuracy (sensitivity and specificity) of the MMP-8 levels by chair-side test kit were compared with ELISA at baseline and 3 mo after scaling and root planing among the study population.

RESULTS: The novel chair side test detected MMP-8 levels in accordance with ELISA which at baseline were higher in Group 2 and Group 3 as compared to controls ($P < 0.05$), and these enzyme levels decreased after therapy ($P < 0.05$). The chair-side test could differentiate healthy, gingivitis and periodontitis. The detection accuracy of the chair-side test strip were on par with ELISA (sensitivity 92.9% and specificity of 100%) which were statistically significant ($P < 0.05$). A desire to arouse interest about periodontal health and maintenance in the Indian population provided a strong rationale for us to develop our chair-side test strips to suit our economy. Moreover, this was the first ever effort to develop and validate a chair-side test strip to detect MMP-8 levels in the Indian population. This test can be used on a large scale in private dental practice for the early detection of disease, tapping the sites at risk for disease, alongside helps in patient education and motivation for maintenance.

CONCLUSION: This study shows that the novel chair side test kit detects MMP-8 levels a biomarker of periodontal disease progression accurately making it a good chair side diagnostic tool. Further, it is cost effective and time saving which can make it applicable in private dental practice on a large scale for the early detection of periodontal disease.

Key words: Chair-side test; Chronic periodontitis; Gingival crevicular fluid; Matrix metalloproteinase-8; Periodontal health

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INTRODUCTION

Periodontal diseases are chronic inflammatory diseases of

the supporting structures of the teeth. Though periodontitis is often triggered by periodontopathogens, its clinical outcome is highly influenced by the host local immune response[1]. In view of the irreversible nature of progressive periodontitis, an early diagnosis and treatment of this disease is important. An early diagnosis will help prevent further irreversible loss of connective tissue attachment of teeth and adjacent alveolar bone associated with periodontal disease[2,3]. It is a well-established fact that the host immune products in periodontitis are synthesized locally and appear within the gingival crevicular fluid (GCF). This makes GCF ideal for obtaining diagnostic information of periodontal health or disease status[4]. The markers thus identified include cytokines, prostaglandins, bacterial- as well as host-derived enzymes, and connective tissue-degradation products, alongside bone matrix components that are primarily isolated in the GCF[5].

Matrix metalloproteinases (MMPs) are one such Group of enzymes which play a key role in the mediation of tissue destruction in periodontitis. MMP-8, a collagenase synthesized by neutrophils, is the major metalloproteinase implicated in the degradation and remodeling of the extracellular matrix. MMP-8 has a strong affinity towards type I collagen, which is present in abundance in the periodontal tissues[6]. There is growing evidence which indicate that a predominant association exists between increased GCF collagenase activity and disease progression, as it is extensively distributed in diseased periodontal tissues. A significant decrease in the GCF MMP-8 activity has been demonstrated following successful non-surgical periodontal therapy[7,8]. The potentiality of MMP-8 as a biomarker of periodontal disease progression is evident from literature.

Keeping this in view, the first chair-side, point-of-care dip-stick test based on the principle of immunochromatography utilizing monoclonal antibodies was developed and tested successfully to detect MMP 8 levels in GCF[9]. However, it was not popularized to be used on a large scale in private dental practice, probably because there were no further studies reported in literature evaluating and validating its use on a large sample size; Secondly, cost of the test kit could have escalated with the use of monoclonal antibody.

This fact provides a strong clinical rationale to indigenously develop a point-of-care test utilizing polyclonal antibody which is cost effective compared to monoclonal based test kit for MMP-8 detection with a diagnostic accuracy on par with enzyme-linked immunosorbent assay (ELISA). To overcome this limitation we indigenously developed a novel chair side point of care dip stick test based on the principle of immunochromatography utilizing polyclonal antibody (cost effective) instead of monoclonal antibody to detect MMP-8. The objective of the current study is to validate diagnostic accuracy (sensitivity and specificity) of our indigenously developed a novel chair-side test kit compared to that of ELISA in periodontal health and disease. Further to check the cost-effectiveness to suit the economies of developing countries like India.

MATERIALS AND METHODS

Study population

The study population consisted of 187 subjects (77 males and 90 females), 30-39 years of age, who were screened from the outpatient section of Department of Periodontics, Krishnadevaraya College of Dental Sciences and Hospital, Bangalore and 150 subjects (75 males and 75 females) were recruited for the study that was conducted during April to May 2010.

The following criteria prevented the patients from being included in the study Groups: medically compromised patients requiring prophylactic antibiotics, patients on antibiotic therapy within the last 6 mo, patients who had received any form of periodontal therapy surgical or non-surgical within 6 mo of baseline examination, smokers, pregnant patients, patients with recent orthodontic treatment, pulpal or periapical involvement on the qualifying teeth.

Each subject underwent a full mouth periodontal probing and charting, the subjects were categorized into three Groups based on clinical examination of gingival index (GI) (Loe and Silness, 1963), plaque index (PI) (Turesky Gilmore Glickman modification of the Quigley and Hein plaque index, 1970) and probing pocket depth (PPD) using a UNC15 probe. Fifty subjects with clinically healthy periodontium, mean PI $\leqslant$ 1, mean GI $\leqslant$ 1, no PPD, were included in Group 1. Group 2 (gingivitis Group) consisted of Fifty subjects with gingival inflammation as indicated by the mean PI $\geqslant$ 2, GI $\geqslant$ 2 and the absence of PPD. Group 3 (chronic periodontitis) consisted of fifty subjects with a mean PI $\geqslant$ 2, GI $\geqslant$ 2 and PPD $\geqslant$ 5 mm falling in the category of severe periodontitis as per the classification. Therefore of the patients recruited in the study, Group 1 had individuals with a healthy periodontium, Group 2 included individuals with gingivitis and no attachment loss and those in Group 3 were diagnosed to have severe chronic periodontitis. Groups 2 and 3 were treated with non surgical approach, scaling and root planning was done using area-specific Gracey currets (Hu friedy) and ultrasonic scalers.

Subjects satisfying the above criteria for enrolment were selected consecutively, and ethical clearance for the study was obtained from the institutional ethical review board, Rajiv Gandhi University of Health Sciences, in accordance with the guidelines of Indian council of Medical research. Written informed consent as per the declaration of Helnski 2008 was obtained from those who agreed to participate in the study.

Site selection and collection of GCF

The study was a triple blind prospective cross-sectional study in which clinical examination, Group allocation and sample site selection was performed by one examiner, the

samples were collected on the subsequent day by the second examiner, and a third examiner carried out the post-treatment clinical examination. This was done to ensure masking of the sampling examiner and to prevent contamination of GCF with blood associated with the probing of inflamed sites. One site per subject was sampled, in gingivitis patients, site with most severe clinical inflammatory signs (in gingivitis cases) or greatest amount of probing depth (in chronic periodontitis cases), along with radiographic confirmation of alveolar bone loss, and the same test site was selected for the after-treatment Group.

On the subsequent day, after drying the area with blast of air, supragingival plaque was removed without touching the marginal gingiva to eliminate the possibility of saliva contamination and thereafter GCF was collected. A standardized volume of 3 μL was collected from each test sites using the calibration of colour coded 1-5 μL calibrated volumetric microcapillary pipettes (Sigma Aldrich, St. Louis, MO) with an extracurricular (unstimulated) method. The test site, which did not express any volume of GCF, and microcapillary pipettes suspected of being contaminated with blood and saliva were excluded from the study. In such cases the sample was obtained from the tooth that showed the next highest PPD in the same patient. The GCF collected was transferred to eppendorf tubes containing 0.5 mL of phosphate buffer saline and stored at -70 ℃ until the time of assay.

Though cumbersome this method of GCF collection was adopted as to prevent protein binding to the paper strips and the risk of sample evaporation.

MMP-8 assay

The samples were assayed for MMP-8 levels using commercially available ELISA kit. The assays were conducted according to the manufacturer's instructions. Highly sensitive ELISA kit (Booster Biological technology Co., LTD, Shanghai) was used to detect the enzyme levels in the sample. This kit reported an assay of sensitivity of < 10 pg/mL. In relation to specificity, the manufacturer reported no significant cross reactivity or interference for the ELISA kit. The samples were run in duplicates. The kit made use of biotinylated anti-human MMP-8 antibody and Avidin-Biotin-Peroxidase Complex. Absorbance of the substrate colour reaction was read on ELISA reader using 450 nm wavelengths. The total MMP-8 level was determined in nanograms (ng), and the calculation of the concentration in each sample was performed by dividing the amount of enzyme by the volume of sample (ng/mL).

Fabrication of point-of-care test sticks for chair-side monitoring

The chair-side test was fabricated based on the sandwich ELISA principle. Nitrocellulose membrane of pore size 0.45 μm was cut into small strips. One end of the strip was treated with methanol to make the membrane hydrophilic. Methanol was washed off using 0.01 mol/L phosphate buffered saline (PBS) buffer. 1 μL of a primary polyclonal antibody to human MMP-8 was added to the hydrophilic end of the nitrocellulose membrane and allowed to dry. GCF sample collected was transferred to an eppendorf tube containing 0.5 mL of 0.01 mol/L PBS buffer. The test strip was immersed into this tube to allow the sample to bind the primary antibody. The strip was removed and washed thoroughly in the PBS buffer to remove the unbound sample. The strip was then dipped in an eppendorf tube containing secondary antibody to human MMP-8 conjugated with a peroxidase system for 10-15 min. The strips were washed in the buffer again and transferred to another eppendorf tube to which a colour developing solution was added. The solution turns blue in colour for samples positive for MMP-8. A colour change within 5 min was recorded as +++ (strongly positive), a change between 5 to 10 min was recorded as ++ (moderately positive) and a change in colour after 15 min was recorded as + (weakly positive). The individual reading the test results was unaware of the ELISA results to eliminate bias and ensure blinding.

Statistical analysis

Analysis of variance was used to compare all variables between groups and a P value of $\leqslant 0.05$ was considered to be statistically significant. SPSS version 13 was used for all the analysis.

RESULTS

Recruitment of subjects for the study started in the first week of April 2010, recruitment ended by the end of the second week of April. 187 subjects (77 males and 80 females), 30-39 years of age, were screened from the outpatient section of Department of Periodontics, Krishnadevaraya College of Dental Sciences and Hospital, Bangalore. Of the 187 individuals screened only one hundred and fifty subjects (75 males and 75 females) were available for analysis. GCF MMP-8 levels measured by enzyme linked immune sorbent assay were able to distinguish sites with periodontitis from those with gingivitis and healthy sites. All samples in each Group tested positive for MMP-8. The highest mean concentration of MMP-8 was obtained in Group 3 (1948.65 ± 916.44 mg/mL), and the lowest mean concentration was obtained in Group 1 (96.90 ± 30.88 mg/mL). The mean MMP-8 concentration for Group 2 (797.94 ± 185.60 mg/mL) was intermediate between the healthy and periodontitis sites. GCF MMP-8 levels > 1 mg/mL especially helped to differentiate the periodontitis from gingivitis and healthy sites, and 1 mg/mL was used as cut-off point in the chair-side test as previously reported (Tables 1 and 2).

A significant improvement in clinical parameters was observed after treatment (Table 3). κ statistics were performed to know the degree of agreement between the test stick and ELISA results. κ value was 0.959, indicating

Table 1 Descriptive data showing comparison of enzyme-linked immunosorbent assay and test stick results

Visit	Group	Test stick results	MMP-8 (mg/L)		Total
			> 1	≤ 1	
Baseline	Group 1	Positive		1	1
		Negative		49	49
		Total		50	50
	Group 2	Positive	4	0	4
		Negative	8	38	46
		Total	12	38	50
	Group 3	Positive	30	2	32
		Negative	5	13	18
		Total	35	15	50
3 mo	Group 1	Positive		1	1
		Negative		49	49
		Total		50	50
	Group 2	Negative		50	50
		Total		50	50
	Group 3	Positive	13	0	13
		Negative	0	37	37
		Total	13	37	50

MMP: Matrix metalloproteinase.

Table 2 Test strip results at baseline and three month follow-up

Visit	Group	Site	Test result of site	≤ 1 mg/L	> 1 mg/L
Baseline	Group 1	50	Positive	1	0
			Negative	49	0
	Group 2	50	Positive	0	4
			Negative	38	8
	Group 3	50	Positive	2	30
			Negative	13	5
3rd mo	Group 1	50	Positive	0	1
			Negative	0	49
	Group 2	50	Positive	0	0
			Negative	0	50
	Group 3	50	Positive	0	13
			Negative	37	0

a good agreement between the two tests at both baseline as well as the third month follow-up.

DISCUSSION

MMP-8 or collagenase-2 is one of the central biomarkers in the breakdown of periodontal connective tissue during the transition from health to disease[7,10-13] besides it has been found to be a potential candidate for use in diagnostic aids[14,15].

A triple blind prospective study was done to gain an insight into the diagnostic accuracy of the MMP-8 chair-side test and check its cost-effectiveness for application on a large scale. A possible role of MMP-8 as a mediator of periodontal inflammation and a comparison of the levels of MMP-8 in GCF among the three study Groups namely Group1, Group 2, and Group 3 and after treatment in Group 2, and Group 3 was assessed using our chair-side point of care test as well as ELISA. Since there

Table 3 Changes in clinical parameters from baseline to three months following scaling and root planning *n* (%)

Visit	Bleeding on Probing	Probing pocket depth		Total
		≥ 5 mm	< 5 mm	
Baseline	Negative	8 (30.8)	8 (33.3)	16 (32.0)
	Positive	18 (69.2)	16 (66.7)	34 (68.0)
	Total	26 (100.0)	24 (100.0)	50 (100.0)
3 mo	Negative		43 (86.0)	43 (86.0)
	Positive		7 (14.0)	7 (14.0)
	Total		50 (100.0)	50 (100.0)

have been reports on age-dependent changes in inflammatory mediators, we selected subjects in the age Group of 30-39 years to control the influence of age on the levels of MMP-8[16]. Further, a single site was selected for sample collection from each participant, which precluded the pooling of samples from multiple sites. Unstimulated samples were collected as an increase in vascular permeability of the blood vessels following gingival stimulation has been reported[17], suggesting that the levels of MMPs in GCF could be influenced by stimulation in sampling.

To the authors' knowledge, this study was the first of its kind to investigate the GCF levels of MMP-8 in the Indian population. Furthermore, this was the first attempt to tailor a chair-side diagnostic test to detect these enzyme levels in Indian population and suit their economic status as well. Though previous studies have successfully designed a chair-side test it had few shortcomings, firstly, the cost of the test strip which was escalated due to the use of monoclonal antibodies. Second, there was no data on post-treatment assessment of MMP-8 levels using the chair-side test which would have been of great help in patient education and motivation, and to identify the site at risk for disease progression.

The main finding of our study was that a strong association exists between the MMP-8 levels and the degree of inflammation as indicated by the changes in clinical parameters and the enzyme levels. The chair-side test strips also showed good agreement in this accord as depicted by the κ values. The specificity and sensitivity of the test strip were found to be good (sensitivity 92.9% and specificity of 100%).

Test strips were fabricated using polyclonal antibodies instead of the monoclonal antibodies previously used, this cut down the cost of the chair-side test almost threefold. Moreover, the specificity and sensitivity remained on par with the previously designed test, this further confirmed that the specificity and sensitivity obtained with either polyclonal or monoclonal antibodies remained almost the same[18] validating our results.

Even though ELISA is a highly sensitive assay and is widely used to detect various biomarkers to aid in diagnosis of various diseases there were certain limitations of ELISA, like technique sensitivity, the time consuming, a delay in providing results to the patients and the cost

involved favoured the fabrication of an easy to use chair-side test strip.

Our chair-side test strip confirmed that it serves as a good diagnostic tool and helps in early detection and maintenance of patients. However, due to the cross-sectional setting of the present study no definite conclusion can be drawn in this regard. To overcome this limitation of the present study, more number of multicenter trials need to be carried out with larger sample sizes. In the near future the chair-side tests could help in the diagnosis of at risk sites in periodontal patients as well as in the early detection and control of periodontal disease in patients at risk due to various systemic and environmental factors. Currently, a randomized controlled trial on a large sample size is being carried out by our organization to tap the at risk population with our new chair-side diagnostic test strip for MMP-8 detection. Apart from this another trial using the chair-side MMP-8 test is being used to detect MMP-8 levels in saliva samples as well to make it less cumbersome and more cost effective.

In short, it can be said that MMP-8 levels reflects the levels of inflammation in the tissues and this can be maintained at low levels with good patient education and regular maintenance. Currently, ELISA is widely used to detect biomarkers of various diseases. But it has some limitation like technique sensitivity and time consuming and cannot be adopted as a chair-side diagnostic aid. The quest to develop a rapid chair-side diagnostic aid with high validity prompted us to fabricate an indigenous chair-side point of care test.

COMMENTS

Background

Periodontitis is a complex, multifactorial disease, whose progression depends on the interplay between periodontopathogens, environmental factors and the host response. Matrix metalloproteinase-8 (MMP-8) is one of the major enzymes of host response to influence the degradation of the periodontal connective tissues. Many studies have proved this role of MMP-8, a chair-side test kit was also fabricated but was not popularized.

Research frontiers

The detection of MMP-8 by various methods has been carried out regularly. However, a point- of-care chair-side test with diagnostic accuracy on par with the regular methods like enzyme-linked immunosorbent assay as well as economic stability was lacking in developing countries like India. In this study, the authors demonstrated the accuracy of an indigenously prepared chair-side test which was suitable for large scale private practice use as well.

Innovations and breakthroughs

This study confirmed that a strong association exists between the MMP-8 levels and the degree of inflammation as indicated by the changes in clinical parameters and the enzyme levels. The chair-side test strips also showed good agreement in this accord as depicted by the κ values. The specificity and sensitivity of the test strip were found to be good (sensitivity 92.9% and specificity of 100%). Therefore it serves as a good diagnostic tool and helps in early detection and maintenance of patients.

Applications

Thus the indigenously fabricated test can be used along chair-side to detect the MMP-8 levels and identify at risk sites and patients thus aiding in an early diagnosis and good maintenance of periodontal patients.

Terminology

MMPs are matrix metalloproteinases, which represent a Group of Zinc dependent enzymes involved in connective tissue degradation. Of these MMP-8 is the major enzyme with an affinity towards collagens.

Peer review

This study is interesting and the results are useful to the readers. The authors concluded that the novel chair side test kit detects MMP-8 levels a biomarker of periodontal disease progression accurately making it a cost effective and time saving diagnostic tool. It really adds new information. It is a prospective well designed research study with interesting results. The paper is well written with appropriate structure. Thus, it could be accepted for publication.

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