Materials and Methods

3.1 Study Subjects:

The study included three groups of the subjects (i) Controls: 50 healthy individuals (controls for MM study), and 50 females individuals (controls for cervical cancer study) with no major illness in the recent past. (ii) 50 MM untreated cancer patients and (iii) 50 untreated cervical cancer patients. The patients were enrolled from out patient department of GCRI, Ahmedabad. All the subjects were personally interviewed using the questionnaire designed according to the inclusion and exclusion criteria for the study. The information related to personal details, detailed history of any disease in past as well as family history of cancer, clinical details at the time of diagnosis, routine analysis and clinical reports at the institute and outside clinics etc. were obtained.

Table-1: Clinical details of controls and MM patients

Controls (MM study)	**N=50**
Age: Male Female	Mean (Range) 54 years (35-77 years) 54 years (40-75 years)
Sex: Male Female	N=33 N=17
MM patients	**N=50**
Age: Male Female	Mean (Range) 54 years (35-77 years) 54 years (40-75 years)
Sex: Male Female	N=33 N=17
Presence of M-protein	N=29
Absence of M-protein	N=21

Table-1 shows clinical details of MM patients. Diagnosis of the disease was based on clinical, biochemical, radiological and biopsy examinations (Major and Minor criteria) of the patients. Mean age of the patients was 54 years.

Table-2 shows clinical details of cervical cancer patients. Diagnosis of the disease was based on screening, clinical, biochemical, haematological radiological and histopathological examinations of the patients. Staging was done according to the AJCC classification (2002). Mean age of the patients

was 46 years. Majority of the patients had SCC, advanced malignant disease and moderate tumour differentiation.

Table-2: Clinical details of controls and cervical cancer patients

Controls (Cervical cancer study)	**N=50**
Age (Range)	43.5 years (33-58 years)
Cervical cancer patients	**N=50**
Age (Range)	45.6 years (20-70 years)
Site: Cervix	
Histopathology:	
Squamous cell carcinoma (SCC)	40
Epidermoid cell carcinoma (ECC)	02
Unknown	08
Stage of the disease (TNM)	
Stage I	02
Stage II	14
Early stage [Stage I + Stage II]	16
Stage III	26
Stage IV	08
Advanced stage [Stage III+ Stage IV]	34
Pathological tumour differentiation	
Well	02
Moderate	29
Poor	06
Moderate to Poor	09
Unknown	04

3.2 Blood sample collection and processing:

Instutional approval for the study was obtained. Prior consent was taken from all the subjects to participate in the study. Total two hundred blood samples were collected from the subjects by venipuncture in plain sterile vaccuttes. Blood samples from the MM and cervical cancer patients were drawn before initiation of anticancer therapy. Blood samples were allowed to form clot at room temperature and centrifuged at 3000 rpm for 15 minutes. The sera were stored at -20^{0}C until analysis.

The parameters analysed from serum samples were as follows:

No.	Parameters	Controls	MM patients	Cervical cancer Patients
i	**Total proteins**	Total Proteins	Total Proteins	Total Proteins
ii	**Glycoprotein constitutes**	TSA Fucose Hexoses MP	TSA Fucose Hexoses MP	TSA Fucose Hexoses MP
iii	**Immunoprofiling**	(IgG, IgA, IgM)	(IgG, IgA, IgM)	------
iv	**Agarose gel electrophoresis**	Albumin Alpha-1 Alpha-2 Beta Gamma A:G Ratio	Albumin Alpha-1 Alpha-2 Beta Gamma A:G Ratio	------
v	**Native-PAGE- Total protein**	UnLMW Prealbumin Albumin Alpha Beta Gamma	UnLMW Prealbumin Albumin Alpha Beta Gamma	UnLMW Prealbumin Albumin Alpha Beta Gamma
vi	**Native-PAGE-Glycoprotein**	UnLMW Prealbumin Albumin Alpha Beta Gamma	UnLMW Prealbumin Albumin Alpha Beta Gamma	UnLMW Prealbumin Albumin Alpha Beta Gamma
vii	**SDS-PAGE**	Protein separation	Identification of protein Isolation of M-protein	Identification of Protein
viii	**Proteome Analysis**	------	Isolated M-protein	-------
ix	**M-protein glycoprotein constitutes**	------	TSA Fucose Hexoses	------
x	**Gelatinases**	MMP-2 MMP-9	MMP-2 MMP-9	MMP-2 MMP-9
xi	**Inhibitiors of gelatinases**	TIMP-1 TIMP-2	TIMP-1 TIMP-2	TIMP-1 TIMP-2

3.3 Reagents, Kits and Instruments:

The Kits, chemicals and reagents were obtained from BIO-RAD, Amersham, Pharmacia, Calbiochem, Qualigens, Merck and SISCO research laboratory. Spectrophotometric analysis was carried out by Backman DU-640 scanning spectrophotometer. Diffu plates for immunoprofiling were purchased from Biocientifica SA. Agarose gel electrophoresis was performed with the set-up purchased from Banglore, Genei (India). The set-up for native and SDS-PAGE was purchased from BIOTECH yercaud set-up (India). Electro transfer unit was used for the M-protein isolation (Amersham Pharmacia). Hybond ECL Nitrocellulose membranes were purchased from Amersham pharmacia. ELISA Kits for gelatinases and their inhibitors were obtained from R&D systems (USA). PROTEAN IEF cell (1^{st} dimension) and PROTEAN II XL (2^{nd} dimension), gel documentation system and PDQuest, the discovery series^{TM} software (BIO-RAD Laboratories, USA) were used for proteome analysis.

All the parameters were studied by highly sensitive and specific methods as briefly described as follows:

3.4 Spectrophotometric Analysis

Serum Total Protein

Serum total proteins were estimated by Biuret method (Wootton IDP, 1964).

Principle: Under alkaline condition protein containing two or more peptide bonds join together directly or through a single carbon or nitrogen atom and those which contain (-CONH-) complexes with alkaline $CuSO_4$ solution give a blue or purple coloured complex which is read spectrophotometrically at 540 nm.

Reagents:

1) Biuret reagent: 8.8 gm sodium hydroxide (NaOH), 5.0 gm KI, 9.0 gm sodium-potassium tartarate and 3.0 gm $CuSO_4.5H_2O$ were dissolved in 1000 ml of double distilled water (D.D.W).

2) Protein standard: 0.2% Bovine Serum Albumin (BSA) was prepared in 0.9 % NaCl and stored at -20^{0}C.

Procedure:

- 1.45 ml of D.D.W was mixed with 0.05 ml of serum.
- 2.5 ml of biuret reagent was mixed properly and kept in boiling water bath for 10 minutes at 37^0 C for the development of purple colour.
- The colour intensity was read at 540 nm.

Total Sialic Acid (TSA)

Serum TSA levels were estimated following thiobarbituric acid method modified by Skoza and Mohos, (1976).

Principle: Periodic acid oxidizes sialic acid to formyl pyruvic acid and when formyl pyruvic acid is heated with thiobarbituric acid, it produces colour. The intensity of colour is enhanced when it reacts with chromophore of dimethyl sulphoxide. The colour intensity is directly proportional to sialic acid content, which is measured spectrophotometrically at 549 nm.

Reagents:

1) Standard solution: 20 µg N-acetyly Neuraminic acid in 1.0 ml D. D. W.
2) 0.025 M Periodic acid in 0.125 N HCl
3) 10% Trichloro acetic acid (TCA)
4) 2% Sodium arsenite in 0.5 N HCl
5) 6% Thiobarbituric acid (TBA): pH 10 with concentrated NaOH.
6) Dimethyl sulphoxide (DMSO)
7) 0.85% Sodium chloride (Nacl)
8) 1 N H_2SO_4

Procedure:

- 0.1 ml of serum was mixed with 0.8 ml of NaCl. 0.1 ml of 1N H_2SO_4 and heated at 80^{0}C for 1 hour to hydrolyze the serum. Then proteins were precipitated using 10% TCA. Protein precipitates were removed by centrifugation at 3000 rpm for 10 minutes.
- 0.25 ml of 0.025 M periodic acid was added to oxidize the sugar.

- After incubation for 30 minutes at 37^0C, 0.2 ml of sodium arsenite was added immediately.
- 0.5 ml of TBA was added to the mixture and kept in boiling water bath for 7.5 minutes for the development of colour.
- 1.5 ml of DMSO was added to enhance the colour development and finally O.D. was read at 549 nm.

Fucose

Serum fucose levels were estimated with cysteine-sulfuric acid reagent (Winzler, 1955).

Principle: Fucose content is determined by the reaction of methyl pentoses using the colour formed by cysteine reagent after precipitating the proteins with ethanol and heating with sulfuric acid. In order to correct for colour developed by other sugars, the O.D. is measured at two wavelengths.

Reagents:

1) 95 % Ethanol
2) 0.2 N NaOH
3) H_2SO_4 : H_2O Mixture: 6:1
4) Cysteine reagent: 0.3 g of cysteine hydrochloride was dissolved in 10 ml of D. D. W.
5) Standard Solution: 50 µg L(-) fucose was dissolved in 1 ml D. D. W.

Procedure:

- 0.1 ml serum was mixed with 5 ml 95 % ethanol in a test tube. It was centrifuged for 15 minutes at 2500 rpm decanted and precipitates were suspended in 3 ml ethanol.
- The suspension was again centrifuged at 2500 rpm for 10 minutes. After the centrifugation the supernatant was discarded.
- The precipitaed proteins were dissolved in 2 ml 0.2 N NaOH. 0.5 ml protein solution was taken in 2 tubes.
- 4.5 ml of H_2SO_4 : H_2O mixture was added in both the tubes boiled for 3 minutes and cooled.

- ❖ 0.1 ml cysteine-sulfuric acid reagent was added in one of the test tubes. Both test tubes were vortexed.
- ❖ O.D. read at 396 nm and 430 nm after 1 hour against a reagent blank.

Seromucoid Fraction: Mucoid Protein and Hexoses

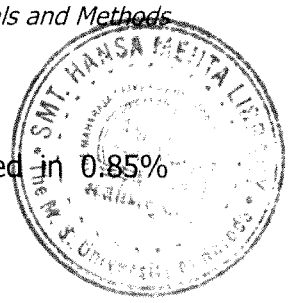
Serum Hexoses was estimated following Orcinol method (Winzler et al, 1955).
Principle: Serum seromucoid fraction is soluble in 1.8 M perchloric acid and it precipitates with 5% phosphotungstic acid. The concentration of the fraction is expressed in terms of its hexoses and protein content.

Mucoid Proteins: MP levels were estimated using Folin Ciocalteu (F.C.) Reagent (Winzler et al, 1955).
Principle: (Lowary et al, 1951). When the F.C. reagent (a mixture of sodium tungstate, molybdate, and phosphate), together with a copper sulphate mixed with protein solution, a blue-purple colour is produced which is read at 750nm. The lowary method is highly sensitive.

Hexoses: Hexoses contents are measured by the reaction with Orcinol-Sulfuric acid reagent. A colour produced is directly proportional to hexoses content, which are measured by spectrophotometrically at 540 nm.

Reagents:

1) Galactose-Mannose standard solution (0.2 gm/ml): 0.1gm of galactose and mannose were dissolved in 1 ml of D.D.W.
2) 0.85% NaCl
3) 1.8 M Perchloric acid
4) 5% Phosphotungstic acid in 2 N HCl
5) 0.1 N NaOH
6) 95% Ethanol
7) 1.6% Orcinol
8) 1% Sodium-potassium tartarate
9) 0.5% Cupric sulphate
10) F.C. reagent



11) 2% Sodium carbonate in 0.1 N NaOH
12) Standard protein solution: 50 µg/ml BSA solution prepared in 0.85% NaCl was used as standard protein solution.

Preparation of reagents:

1) Orcinol-H_2SO_4 Reagent:

Reagent A: 60% H_2SO_4 and Reagent B: 1.6% Orcinol

Orcinol-H_2SO_4 Reagent: 7.5 ml reagent A + 1 ml reagent B

2) Reagent C for MP:

Reagent A: 2% Sodium carbonate in 0.1 N NaOH.

Reagent B: 0.5% Cupric sulphate in 1% Sodium-potassium tartarate

Reagent C: 50 ml of Reagent A + 1 ml of Reagent B.

Procedure:

- 0.25 ml of serum was added in 2.25 ml of 0.85% NaCl in a test tube and 1.25 ml of perchloric acid was added drop wise while shaking the tube.
- The sample was filtered within 10 minute through Whatman filter paper no: 44. 2.5 ml of filtrate was taken in another tube and 0.5 ml of phosphotungstic acid was added.
- After 10 minutes, the solution was centrifuged at 2500 rpm for 10 minutes and the supernatant was discarded.
- The precipitates were washed with 1.0 ml 95% ethanol then centrifuged and supernatant was again discarded.
- The residue was dissolved in 1.0 ml of 0.1 N NaOH and hexoses as well as protein contents of the solution were measured.

MP estimation:

- 0.05 ml of seromucoid fraction and 0.45 ml of D.D.W were added in a test tube.
- 2.25 ml of (Reagent-C) was added into the tube and incubated for 10 minutes at room temperature.
- 0.25 ml of F.C. reagent was added and kept at room temperature for 30 minutes. After incubation, O.D. was observed at 750 nm.

Hexose estimation:

- 0.5 ml of seromucoid fraction was taken in a test tube and 5 ml of Orcinol-H_2SO_4 reagent was added, the tube was kept in boiling water bath for 15 minutes, solution was cooled at room temperature and O.D. was taken at 540 nm.

For all spectrophotometric assays including serum protein, TSA, fucose, hexoses and MP, the concentrations were calculated using standard curve obtained from standards. The following standard formula was used for the estimation.

Calculations:

$$\text{Concentration} = \frac{\text{OD of test} \times \text{Conc. Of std. in mg} \times 100}{\text{OD of Standard} \times \text{Volume Of test}}$$

3.5 Electrophoretic Analysis: Protein fractions and profiling were studied from serum samples using following methods:

- Agarose gel electrophoresis
- Poly-acrylamide gel electrophoresis (PAGE): Native-PAGE and SDS-PAGE

Agarose Gel Electrophoresis

Principle: Serum proteins were separated on 0.5% agarose gel electrophoresis by standard protocol (Martin and Franglen, 1954).

Reagents preparation:

1) Barbitone buffer (pH 8.6): 0.866 gm calcium lactate, 9.1 gm sodium barbitone and 1.33 gm barbituric acid were dissolved in 1000 ml of D.D.W.
2) 1% Amido Black
3) 0.5% Agarose
4) 3% Acetic acid

Procedure:

- Serum sample was loaded using a Whatman filter paper strip (7.5 cm × 2.7 cm) placed at the 1/3rd distance of the slide towards cathode. A

constant current of 4mA/100V was applied per slide and electrophorosed for 60 min. After completion of the run, the slide was dipped into methanol and dried in an incubator.

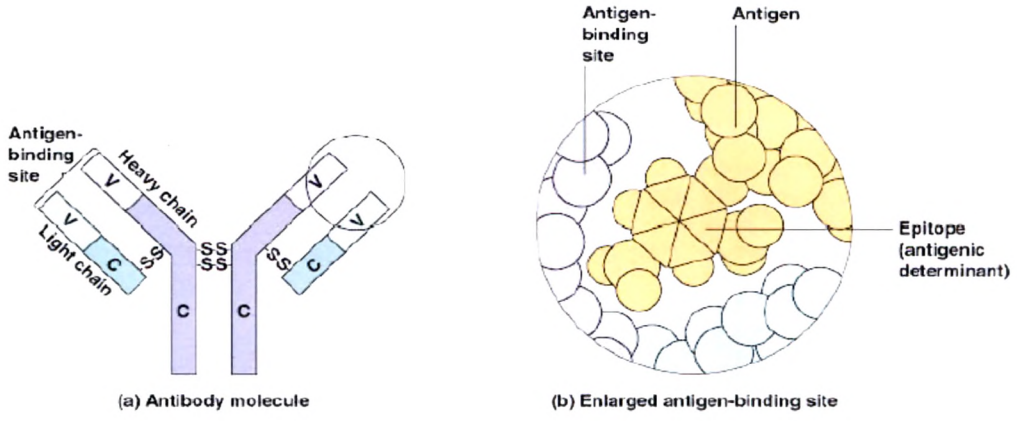
- The slide was stained with 1% amido black and destained with 3% acetic acid until a clear background was obtained.
- Densitometric quantification of each protein band was done (BIO-RAD, Model GS-700 image densitometer) gel documentation system.

3.6 Serum Immunoprofiling (IgG, IgA and IgM)

Immunoglobulins were studied from serum samples using (Diffu-Plate, Biocientifica S.A.) by radial immunodiffusion method (Mancini et al, 1965).

Principle: The immunodiffusion techniques were used to detect the antigen and antibody precipitation reaction. An immunoprecipitation in agarose between an antigen and its homologous antibody is performed by incorporating one of the two immune reactants (usually antibody) uniformly throughout a layer of agarose gel, and then introducing the other reactants (usually antigen) into wells, duly punched in the gel. Antigen diffuses radially out of the well into the surrounding gel-antibody mixture, and a visible ring of precipitation forms where the antigen and antibody are reacted. The diameter of the ring depends on the concentration of immunoglobulin (**figure: 1**).

Figure-1: Schematic diagram shows the correlation between antigen and antibody by the precipitation reaction



Kits: Radial immunodiffu plates (IgG, IgA and IgM)

Procedure:

- The plate from the 4^0C storage were opened and kept at room temperature for 5 minutes at room temperature, allowing the condensation to evaporate.
- The wells were filled with 5µl of serum or control sample.
- The plate was kept with wet cotton to avoid agarose dehydration and closed tightly.
- The plate was allowed to stay as per the instructions in kit inserts.

Results evaluation: End point of diffusion was indicated by a sharp precipitation ring. It was achieved when incubation time is finished. The ring diameter was measured and values were calculated.

3.7 PAGE (Native/SDS)-Serum Total Protein and Glycoprotein Profiling

Serum proteins were separated on 10% native-PAGE by standard protocols (Sambrook, Russell 2001).

Reagents:

1) 30% Acrylamide: Bisacrylamide (W/V)
2) Resolving Buffer (1.5 M Tris-HCl pH 8.8)
3) Stacking Buffer (0.5 M Tris-HCl pH 6.8)
4) Electrophoresis Buffer pH 8.3
5) 10% Ammonium per Sulfate (APS)
6) N, N, N', N' Tetramethylene Diamine (TEMED)
7) 0.01% Bromophenol Blue (BPB) in 40% sucrose
8) Saturated Butanol
9) Staining dye: Coomassie Brilliant Blue R-250 (CBB-R-250)
10) Glycoprotein profiling dye: Schiff's reagent
11) Destaining solution

Native-PAGE Protocol (figure: 2):

Composition of 10% resolving gel:

- 4.0 ml of D.D.W, 3.34 ml of 30% acrylamide:bisacrylamide and 2.5 ml of 1.5 M Tris HCl pH 8.8 were mixed properly. This was followed by degassing were for 5 minutes.
- 50 µl of 10% APS and 5 µl of TEMED were added and mixed.
- The gel mixture was immediately poured into gel apparatus and it was allowed for polymerization.

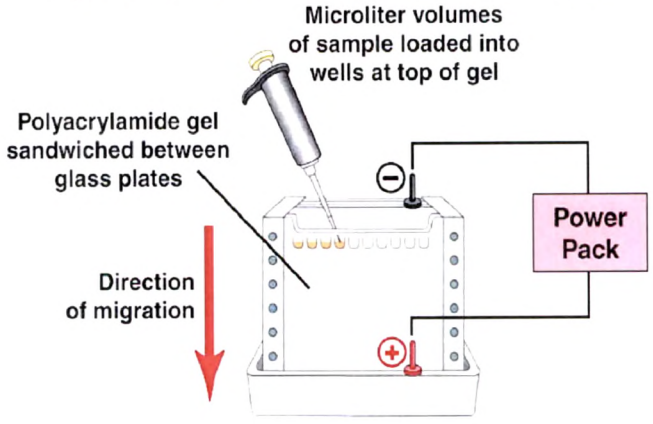
Composition of 5% stacking gel:

- 6.8 ml of D.D.W, 1.66 ml of 30% acrylamide:bisacrylamide and 1.26 ml of 1.5 M Tris HCl (pH 6.8) were mixed properly. This was followed by degassing for 5 minutes.
- 75 µl of 10% APS and 7 µl of TEMED were added and mixed.
- The gel mixture was immediately poured into gel apparatus and it was allowed for polymerization.

Sample preparation for native- PAGE

- 50 µl of serum sample was diluted 1:2 for total protein and glycoprotein analysis with 0.9% NaCl.
- Aliquot calculated based on total protein value and same amount of BPB was added.

Figure-2: Schematic diagram of PAGE



Procedure:

- Mini slab gels were prepared using 10% resolving gel, 5% stacking gel

and electrophoresis buffer pH 8.3.

- For total protein and glycoprotein profiling, 500 µg and 1000 µg of serum proteins were loaded, respectively.
- Electrophoresis was carried out at a constant current of 25V per plate till protein crosses stacking gel then 50V per plate till the tracking dye reached the opposite end. During the electrophoresis process proper cooling (4^0C) was maintained.

Staining for total protein:

- For total protein, the slab gels were taken out and stained with 0.1% CBB-R-250 and destained until a clear background was obtained.
- Gels were scanned and then dried on gelatin sheet for preservation.

Staining for glycoprotein (Fairbanks et al, 1971)**:**

- The gels were immersed in 0.5% (w/v) periodic acid in D.D.W. for 2 hours. They were then transferred into 0.5% (w/v) sodium arsenite solution in 5% acetic acid for 1 hour.
- The gels were transferred and incubated in 0.1% sodium arsenite (w/v) in 5% acetic acid for 20 minutes (repeated twice).
- Fixing was performed in 10% acetic acid (v/v) for 30 minutes. After pouring off the acetic acid. Schiff's reagent was poured in a tightly sealed box till the gels were dipped completely for 2 hours.
- Destaining was done with 0.1% sodium metabisulphite in 0.01 N HCl until the background was clear.
- Densitometric quantification of each protein band was done with (BIO-RAD, Model GS-800 image densitometer) gel documentation system.
- Gels were scanned and then dried on gelatin sheet for preservation.

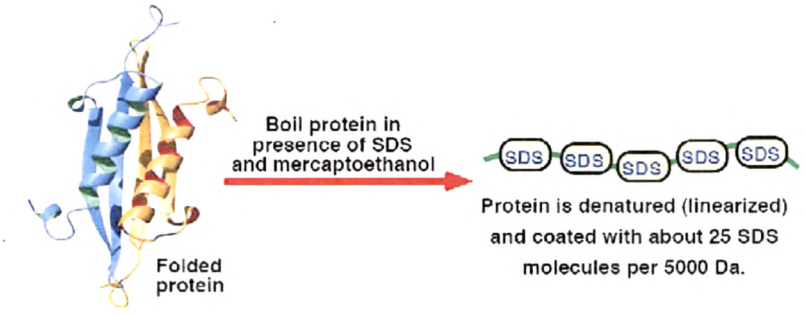
3.8 Total Protein Profiling by SDS-PAGE

Serum proteins were separated on 10% SDS–PAGE by standard protocol (Harlow and Lane, 1999) in a non-denaturing and non-reducing condition. It is useful for monitoring protein purification. This method is based on

separation of protein according to size, it can also be used to determine relative molecular mass of proteins.

SDS-PAGE Protocol (figure: 3):

Figure-3: Schematic diagram of protein linearized in presence of SDS



Composition of 10% resolving gel:

- 4.0 ml of D.D.W, 3.34 ml of 30 % acrylamide:bisacrylamide, 2.5 ml of 1.5 M Tris HCl (pH 8.8) and 75 µl 10% SDS were mixed properly. This was followed by degassing for 5 minutes.
- 50 µl of 10% APS and 5 µl of TEMED were added and mixed.
- The gel mixture was immediately poured into gel apparatus and it was allowed for polymerization.

Composition of 5% stacking gel:

- 6.8 ml of D.D.W, 1.66 ml of 30% acrylamide:bisacrylamide, 1.26 ml of 1.5 M Tris HCl (pH 6.8) and 100 µl of 10% SDS were mixed properly. This was followed by degassing for 5 minutes.
- 75 µl of 10% APS and 7 µl of TEMED were added and mixed.
- The gel mixture was immediately poured into gel apparatus and it was allowed for polymerization.

Sample preparation for SDS PAGE

- 50 µl of serum sample was diluted 1:20 with 0.9% NaCl.
- Sample aliquots were calculated based on total protein value and same amount of sample buffer was added to the sample.
- Samples were heated for 3-4 minutes for denaturation of proteins.

Procedure:

- Mini slab gels were prepared using 10% resolving gel, 5% stacking gel and electrophoresis buffer (pH 8.3).
- Serum sample (75 µg of protein) was loaded in the well. Electrophoresis was carried out at a constant current of 25 V per plate till protein crosses stacking gel then 50 V for rest of the migration till the tracking dye reached the opposite end and proper cooling was maintained. Total protein, the gels were stained with 0.1% CBB-R-250 and destained with the destaining solution.
- Densitometric quantification of each protein band was done with (BIO-RAD, Model GS-800 image densitometer) gel documentation system.
- Gels were scanned and then dried on gelatin sheets for preservation.

3.9 Isolation of M-protein:

Electro-transferred on Nitrocellulose membrane (Amersham Pharmacia).

M-protein isolation consisted of the following steps:

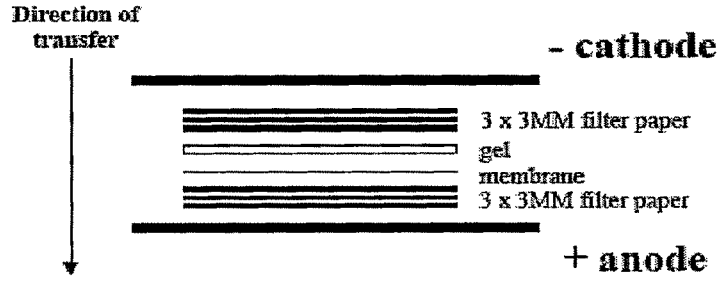
1. Separation of M-protein by native-PAGE.
2. Electro transfer of M-protein bands to Nitrocellulose membrane.
3. Protein eluted from the Nitrocellulose membrane.

Electro transfer of M-protein bands to Nitrocellulose membrane:

Semi dry method was used for transfer of M-protein from gel to membrane. The following procedure was followed:

- Semi-dry blotter: consists of two electrodes, the anode is at the bottom and the cathode is at the top. Negatively charged proteins thus move downwards towards the anode (**figure: 4**).

Figure-4: Semidry method



- Before start the transfer, anode and cathode both were dipped in chilled D.D.W for 2 hours.
- The gel was rinsed briefly in electrophoresis buffer. It was soaked the blotting paper cut to the same size of the separating gel (Nitrocellulose membrane) in towbin transfer buffer for 10 minutes at 4^0C.
- Cut off the stacking gel and soaked the separating gel in the towbin transfer buffer for 10 minutes at 4^0C.
- Soaked 20 pieces of the rough filter paper and Whatman 3MM paper with a size of separating gel in the transfer buffer for 3-4 minutes.
- 10 pieces of pre-wetted rough filter papers followed by 10 pieces of Whatman 3 MM paper and placed them on the anode. 10 pieces of pre wetted membrane on this followed by gel.
- Connected blotting unit with power and carried out electro transfer in the prepared stack for 4 hours at 200mA.

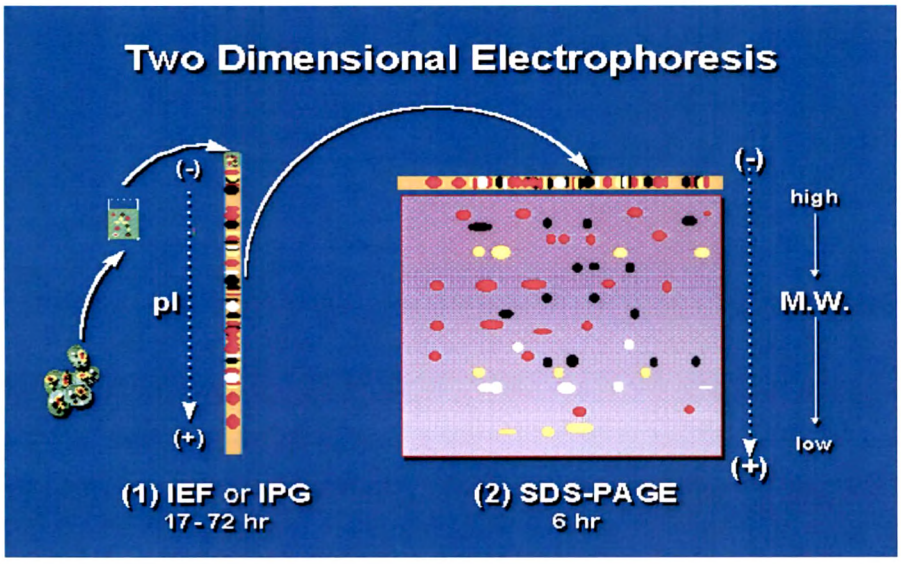
M-Protein elution from the Nitrocellulose membrane

- After blotting procedure, membrane was taken out from the gel and then put into ponceau S dyes for 3-4 minutes to check the protein transfer.
- Decanted dye and washed with D.D.W.
- The membrane was washed frequently to remove dye and M-protein portion was cut and put it into 0.7 ml of elution buffer.
- It was Kept overnight for agitation of M-protein from membrane on an orbital shaker.
- M-protein was isolated; further SDS-PAGE was performed and 2D-PAGE from M-protein fraction was also carried out.
- TSA, fucose and hexoses contents of M-protein fraction were also estimated.

2D-PAGE Approach for M-Protein Analysis: For 2D-PAGE analysis, protein content of isolated M-protein fraction was estimated by Lowry method.

Principle: 2D Gel Electrophoresis technique was performed as suggested by O' Farrell, (1975) (**figure: 5**).

Figure-5: Schematic diagram of 2D-gel electrophoresis



Adapted from www.lmmb.ncifcrf.gov

2D PAGE gel electrophoresis consisted of the following steps:

- ❖ Sample Preparation
- ❖ IPG Strip Rehydration
- ❖ IEF
- ❖ IPG Strip Equilibration
- ❖ SDS-PAGE
- ❖ Protein of 2D Maps
- ❖ Analysis of 2D Maps

Reagents:

All the reagents provided with kit were either lyophilized, concentrated or in ready to use form and were reconstituted according to the kit (BIO-RAD).

1) Ready Prep ™ 2-D starter kit
2) SDS-PAGE Equilibration Buffer-I (with DTT)
   [6M Urea, 0.375 M Tris (pH 8.8), 2% SDS, 20% Glycerol, 2 %(w/v) DTT]
3) SDS-PAGE Equilibration Buffer-II (with Iodo-acetamide)

[6M Urea, 0.375 M Tris (pH 8.8), 2% SDS, 20% Glycerol, 135 mM Iodoacetamide]

4) Ready strip ^{TM} IPG strip, strips 17 cm, PH 3-10NL
5) 30% Acrylamide: Bisacrylamide(w/v)
6) 1.5 M Tris-HCl (pH 8.8)
7) Electrophoresis Buffer (pH 8.3)
8) 10% SDS
9) 10% APS
10) TEMED
11) PROTEAN IEF cell system
12) PROTEAN II XL system
13) Mineral oil
14) Sample: Isolated M-protein

Preparation of Reagents:

1) 30 % Acrylamide: Bisacrylamide
2) Resolving Gel Buffer: (1.5M Tris–HCl, pH 8.8)
3) Electrophoresis Buffer: Tris -glycine Buffer (pH 8.3) 10 % SDS
4) 10% APS
5) TEMED
6) Silver staining: Silver staining developing solution 0.04 % formalin (w/v) and 2 % sodium carbonate (w/v) in water.
7) Saturated Butanol

Sample preparation: The IPG strips (length 17 cm and pH3-10 NL) rehydrated with the sample buffer and isolated M-protein fraction. The composition of sample buffer included detergents, reducing, and denaturing agents. These buffer hydrophobic interactions, hydrogen and disulfide bonds, unwanted aggregations and the formation of secondary structures, which affect protein mobility.

Composition of 10% resolving gel:

- 40 ml of D.D.W, 33.4 ml of 30 % acrylamide:bisacrylamide, 25 ml of 1.5 M Tris HCl (pH 8.8) and 750 µl 10% SDS were mixed properly. This was followed by degassing for 5 minutes.
- 150 µl of 10% APS and 15 µl of TEMED were added and mixed.
- The gel mixture was immediately poured into gel apparatus and it was allowed for polymerization.

Rehydration and sample application (First day protocol)

1) Prepared sample in rehydration sample buffer.
2) Pipetted the total 300µl rehydration volume of each sample as a line along the back edge of a channel in a rehydration/equilibration tray. The line of sample should extend along the whole length of the channel except for about 1 cm at each end. Take care not to introduce any bubbles, which may interfere with the even distribution of sample in the strip.
3) When the M-protein samples have been loaded into the rehydration/ equilibration tray. Peel the coversheet from the ready IPG strip using forceps.
4) Gently placed the strip, gel side down, on the sample. The '+' and the pH range marked on the strip should be legible. Take care not to get the sample onto the plastic backing of the strips, as the gel material will not absorb this portion of the sample. Also take care not to trap air bubbles beneath the strip.
5) Overlaid each of the strips with 2-3 ml of mineral oil to prevent evaporation during the rehydration process. Added the mineral oil slowly by carefully dripping the oil onto the plastic backing of the strips while moving the pipette along the length of the strip. Alternatively, waited approximately 1 hour after starting rehydration, until the strip has absorbed most of the liquid. Then overlaid the strips with mineral oil.
6) Covered the rehydration/equilibration tray with the plastic lid provided and left the tray sitting on a level bench overnight (11-16 hours).

Iso electric focusing- First Dimension (Second day protocol)

1) Place a cleaned, dry PROTEAN* IEF focusing tray onto the lab bench.
2) Used forceps, place a paper wick at both ends of the channels covering the wire electrodes.
3) Pipetted 8 µl of nanopure water onto each wick to wet them. Removed the cover from the rehydration/equilibration tray containing the IPG strips. Forceps were used carefully to hold the strip vertically for about 7-8 seconds and blotted the tip of the strip to the corresponding channel in the focusing tray.
4) Covered each IPG strip with 2-3 ml of fresh mineral oil and place the lid onto the tray.
5) Placed the focusing tray into the PROTEAN IEF cell and closed the cover. Used the temperature 20^0C with a maximum current 50 µA per strip and three steps at different voltage (250v-Ramp-Linear, 10,000 V-Ramp-Linear and 10,000 V-Ramp-Rapid and finally 40,000-50,000 V-hr).
6) Electrophoresis run was started according to focusing protocol conditions.
7) After the completion of run, the IPG strips were stored in -80^0 C until the second dimension was not performed.

Equilibration and second dimension (SDS-PAGE) (Third Day Protocol)

1) Removed the mineral oil from the Ready Strip IPG strips by placing the strips onto a piece of dry filter paper and blotting with a second piece of wet filter paper.
2) 6 ml of equilibration buffer I with DTT to a rehydration/equilibration tray one channel per IPG strip were used. Transferred the blotted Ready Strip IPG strips (gel side up) to the equilibration tray.
3) Placed the tray on an orbital shaker and gently shake for 10 minutes. selected a slow shaker speed to prevent the buffer from sloshing out of the tray.
4) After placing the IPG Strips on the shaker in equilibration buffer I with DTT.
5) At the end of the 10 minutes incubation, discarded the used equilibration buffer I by carefully decanted the liquid from the tray.

6) 6ml of equilibration buffer II with iodoacetamide to a rehydration/equilibration tray one channel per IPG strip were used. After placing the IPG Strips on the orbital shaker for 10 minutes selected a slow shaker speed to prevent the buffer from sloshing out of the tray.
7) During the incubation, melt the agarose solution in a hot water.
8) Filled a 100 ml graduated cylinder or a tube that is the same length as or longer than the IPG strip length with 1X electrophoresis buffer.
9) The SDS-PAGE gels by blotted away any excess water remaining inside the IPG well using Whatman 3 MM or similar blotting paper.
10) Placed an SDS-PAGE gel in a stand. For ideal IPG strip loading, removed an IPG strip from the disposable rehydration/equilibration tray and dip briefly into the graduated cylinder containing 1X electrophoresis buffer.
11) Used a Pasteur pipette or a disposable plastic transfer pipette and place overlay agarose solution on IPG gel.
12) For gels run in the PROTEAN* plus overlaid agarose using the forceps or a spatula. Carefully pushed the strip into the well.
13) 10% poly acrylamide gel was used for the polymerization of gel, using electrode buffer (pH 8.3). IPG strips 17 cm; pH 3-10NL was held in the well.
14) A constant current of 16 mA per gel was applied for 30 minutes and run the sample and increased it up to 24 mA per gel for 5.5 hours accounting to the conditions of BIO-RAD. Proper cooling was maintained at 15^0 C. After the completion of the run, the gels were taken out from the plates.

Silver staining (Shevchenko et al, 1996)**:**

After electrophoresis, the slab gels were stained as follows:

1) The slab gel was fixed in 50% methanol, 5% acetic acid in water for 20 minutes. It was washed for 10 minutes with 50% methanol in water and additionally for 10 minutes with water to remove the remaining acid.
2) The gel was sensitized by incubating for 1 minute in 0.02% sodium thiosulfate then rinsed with two changes of water for 1 minute each.

3) After rinsing, the gel was submerged in chilled 0.1% silver nitrate solution and incubated for 20 minute at 4^0C. After incubation, silver nitrate solution was discarded and the gel slab was rinsed twice with water for 1 minute and then developed in 0.04% formalin in 2% sodium carbonate with intensive shaking.
4) After the developing solution turned yellow, it was discarded and replaced with a fresh portion.
5) After the desired intensity of staining was achieved, development of the gel was terminated by discarding the reagent, followed by washing of the gel slab with 5% acetic acid.
6) Silver-stained gels were stored in 1% acetic acid at 4^0C until analysed.

Image Acquisition and Analysis:

- The 2 Dimension gels were scanned by the GS-800 calibrated densitometer (BIO-RAD) image analysis system. The digitalized images were analysed.

Computer assisted image analysis of 2D-PAGE gels: Computer-assisted image analysis software is an indispensable tool for the evaluation of complex 2D gels. Computer assisted image analysis was consisted of the following steps:

- Select the application
- Select scan area
- Select resolution of the scan
- Calibrate the instrument
- Acquire the image

Spot detection and spot identification:

- PDQuest, the discovery series™ software models protein spots mathematically as 3D Gaussian distributions and uses the models to determine absorption maxima.
- This enables automatic detection and resolution of merged spots. Following this procedure, spot intensities are obtained by integration of

the Gaussian function. The mathematical description of the spots is used both for data reduction and for increasing evaluation speed, since reevaluation of data after an image change takes only fractions of a second.

- The hit rate of automatic spot detection is highly dependent on the quality of the 2-D gels. Correction capabilities of PDQuest software can be used to add undetected spots to the list of spots or to delete spots that arise from gel artifacts.

Data analysis:

- With PDQuest software, all gels of an experiment were viewed as a unit. Compared gels from different experiments, the reference images were compared.
- Each spot is automatically assigned a number so that identical spots have identical numbers.

2D-PAGE analysis:

The 2D analysis approach was as follows,

- Assessment of differentially expressed protein spots.
- Numbering of protein spots in case of many spots on 2D map.
- Determination of PI and MW of spot of interest and other related information.

3.10 Gelatin Zymography for MMP-2 and MMP-9:

Gelatin zymography (pro- and active forms) were performed according to the method of (Lorenzo et al, 1992).

Sample preparation for MMP-2 and MMP-9 (Meisser et al, 2005)**:**

- 50 µl of serum samples, 450 µl of chilled D.D.W. and 20 µl of 1% acetic acid were mixed and vortexed properly.
- The mixture was incubated at 0^0C for 1 hour and centrifuged at 5000 rpm for 10 minutes.

- Then the pellets were dissolved in chilled 0.1 M Phosphate Buffer Saline (pH 7.4). Aliquots of protein were estimated by lowary method (Lowary et al, 1951).

Reagents preparation:

1) **30 % Acrylamide: Bisacylamide (W/V):** 29.2 gm acrylamide and 0.8 gm bisacrylamide were dissolved in 100 ml of D.D.W.
2) **Electrode Buffer (pH 8.3):** Tris- 3.03 gm and glycine- 14.4 gm dissolved in D.D.W. (1X) 1000 ml and adjust the pH 8.3 with concentrated HCl and added 1 gm of SDS.
3) **Resolving Gel Buffer:** 1.5 M Tris–HCl (pH 8.8) – 18.171 gm of Tris in 100 ml D.D.W. and adjust the pH with concentrated HCl and added 0.4 gm of SDS.
4) **Stacking Gel Buffer:** 0.5 M Tris-HCl (pH 6.8) - 6.05 gm of Tris in 100ml D.D.W. and adjust the pH 6.8 with concentrated HCl and added 0.8 gm of SDS.
5) **Sample Buffer (2X):** SDS (4 %), Glycerol (20 %), BPB (0.004 %) and Tris HCl (0.125 M) were mixed in equal amount.
6) **Gelatin Solution:** 0.025 gm/5 ml (0.5 %)
7) **Molecular weight marker** (Calbiochem, USA)
8) **10% APS**
9) **TEMED**
10) **Saturated Butanol**
11) **7.5% resolving gel preparation:** 30% acrylamide:bisacrylamide (2.5 ml), resolving gel buffer (2.5 ml), D.D.W. (4.0 ml) and gelatin solution (1.0 ml) were mixed and degassed for 5 minutes. Then APS (50 µl) and TEMED (4 µl) were added and mixture was pored in gel casting unit and was allowed until the polymerization occurs.
12) **5.5% stacking gel preparation:** 30% acrylamide:bisacrylamide (0.83 ml), stacking gel buffer (0.63 ml) and D.D.W. (3.40 ml) were mixed and degassed for 5 minutes in sonicator then APS (75µl) and TEMED (4 µl) were added and gel was pored for polymerization.
13) **Washing Solution:** 0.25% Triton-x-100 (1 ml/400 ml D.D.W.)

14) **Activation Buffer:** 50 mmol Tris HCl pH 7.5 containing 10 mmol $CaCl_2$, 1mmol $ZnCl_2$, 0.02 % Sodium azide, 1 % v/v Triton- x-100.

15) **Staining Solution:** 0.1 % CBB R-250 in 40% propanol.

16) **Destaining Solution:** 7% Glacial acetic acid.

Procedure:

- Mini slab gels were prepared using 7.5% running gel co-polymerized with gelatin as substrate and 5% stacking gel.
- Sample aliquot equivalent to 50 µg proteins were mixed with equal volumes of sample buffer dye and kept at room temperature for 30 minutes.
- The samples were run under non-denaturing and non-reducing conditions at a constant current of 25 V per plate till protein crosses stacking gel then constant voltage 50 V for rest of the migration till the tracking dye reached the opposite end. During the run the gels were kept at proper 4^0C using cool water circulating system.
- The gels were separated from the glass plates and washed twice with 0.25% Triton-X-100 (100 ml) for 15 minutes each. This step removes excess SDS from the gels.
- The gels were then incubated overnight for 16 hours in presence of activation buffer.
- The activation buffer was decanted and the gels were stained with 0.1% CBB R-250 in 40% propanol for minimum 1 hour.
- The gels were destained in 7% acetic acid. The washing, overnight activation, staining and destaining were done with gentle shaking of the gels on shaker (**figure: 6**).
- When SDS was removed, the gelatinases refold and digest away the gelatin, leaving a clear band (gelatinolytic acticity) against a blue back ground. The band intensity was determined by scanning the gels densitometrically using (BIO-RAD, GS-700 image densitometer) gel documentation system.
- Commercially available pure human pro and active MMP-2 and MMP-9 standards were also electrophoresed to create standard plots.

Figure-6: Schematic diagram of gelatin zymography protocol

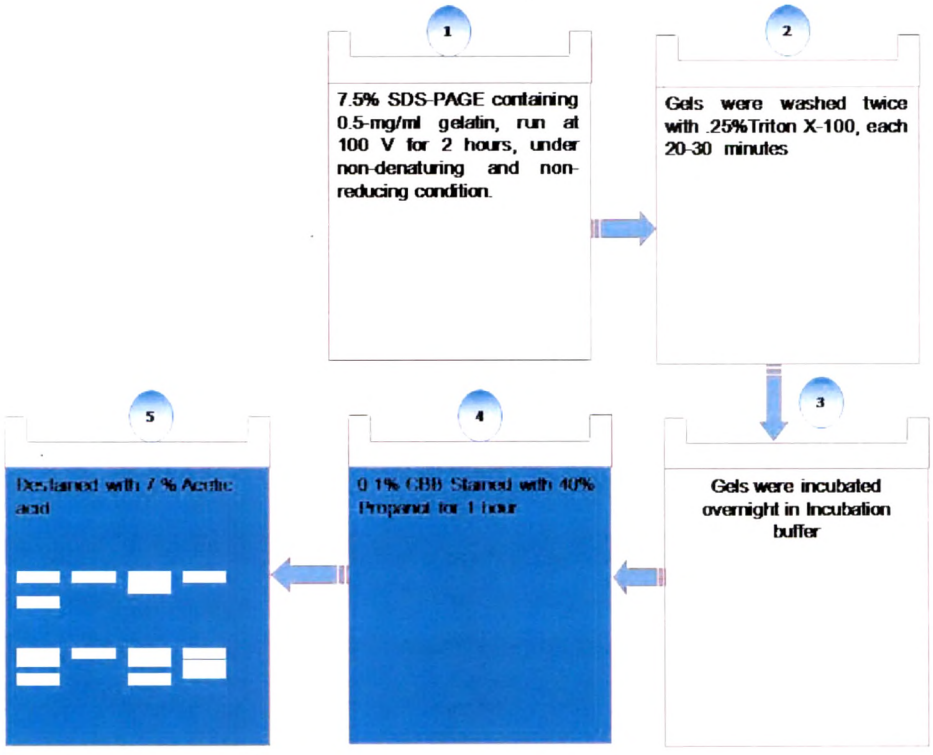
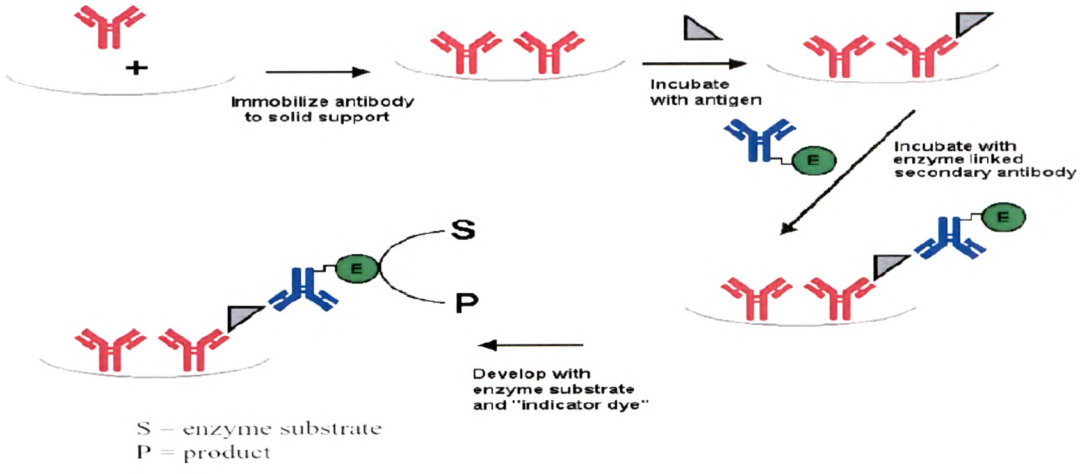


Figure-7: Schematic diagram showing principle of ELISA

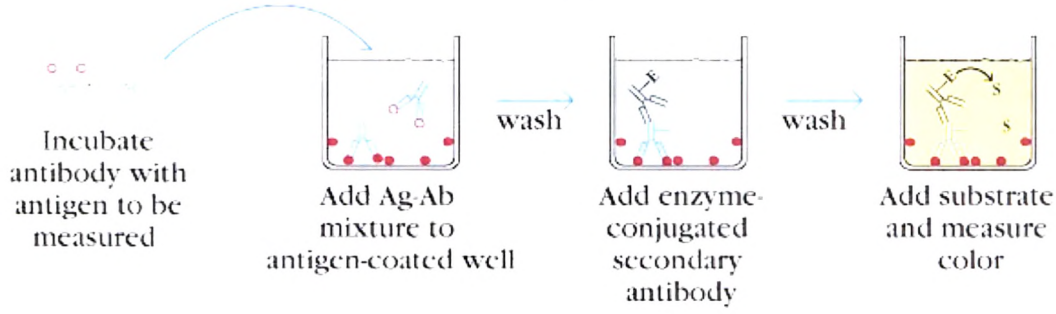


3.11 Principle:

This assay is based on the quantitative sandwich enzyme immunoassay technique (**figure: 7, 8**). A pre-coated polyclonal antibody specific for MMP-2 on a micro plate is used. Standards, controls and samples are pipetted into

the wells, and MMP-2 is bound by the immobilized antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for MMP-2 is added to the wells. Following wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the total MMP-2 (pro and/or active) bound in the initial step. The intensity of the colour is measured.

Figure-8: Schematic diagram of ELISA protocol



Sample preparation:

Serum samples were diluted 1:10 using calibrator diluent provided in kit.

Reagent Preparation:

All the reagents provided with kit were either lyophilized, concentrated or in ready to use form and were reconstituted according to the instruction manual.

1) **Total MMP-2 micro plate:** 96 well polystyrene micro plate coated with a polyclonal antibody against human MMP-2.
2) **Total MMP-2 conjugate:** Polyclonal antibody against human MMP-2 conjugated to horseradish peroxidase, with preservatives.
3) **Assay diluent:** Buffered protein based with preservative.
4) **Calibrator diluent:** Buffered protein based with preservative.
5) **Colour reagent A:** Stabilized hydrogen peroxide.
6) **Colour reagent B:** Stabilized chromogen (Tetramethylbenzidine).
7) **Stop solution:** 2 N sulfuric acid.
8) **Wash buffer:** 21 ml of a 25-fold concentrated solution of buffered surfactant with preservatives.

9) **Substrate solution:** Colour reagent A and B were mixed in equal volumes in dark and were used within 15 minutes.

10) **Total MMP-2 standard:** MMP-2 standards were reconstituted with 1 ml of D.D.W to produce a stock solution of 100 ng/ml.

Preparation of working standards:

- 200 µl of calibrator diluent was pipetted into each tube.
- 200 µl of stock solution was pipetted in first tube to produce working standard of 50 ng/ml.

Assay Procedure

Prepared reagents, samples and standards as instructed.

⇩

Added 100 µl of assay diluent to each well.

⇩

Added 50 µl of standard, control or sample to each well and incubated 2 hours on the horizontal orbital microplate shaker at room temperature.

⇩

Aspirated and washed 4 times.

⇩

Added 200 µl of conjugate to each well and incubated 2 hours on the shaker at room temperature.

⇩

Aspirated and wash for 4 times.

⇩

Added 200 µl of substrate solution to each well, protect from light and incubated half an hour on the benchtop.

⇩

Added 50 µl of stopping solution to each well.

⇩

Read at 450 nm within half an hour (λ correction 540 or 570 nm) on ELISA plate reader.

Total MMP-9 ELISA

Principle:

This assay is based on the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for MMP-9 has been pre-coated on a micro plate. Standards, controls and samples are pipetted into the wells, and MMP-9 is bound by the immobilized antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for MMP-9 is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the total MMP-9 (pro and/or active) bound in the initial step. The intensity of the colour is measured.

Sample preparation:

Serum samples were diluted 1: 80 using calibrator diluent provided in kit.

Reagent Preparation:

All the reagents provided with kit were either lyophilized, concentrated or in ready to use form and were reconstituted according to the instruction manual.

1) **Total MMP-9 micro plate:** 96 well polystyrene micro plate coated with a mouse monoclonal antibody against human MMP-9.
2) **Total MMP-9 conjugate:** Polyclonal antibody against human MMP-9 conjugated to horseradish peroxidase, with preservatives (ready to use).
3) **Assay diluent:** Buffered protein based with preservative.
4) **Calibrator diluent:** Buffered protein based with preservative.
5) **Colour reagent A:** Stabilized hydrogen peroxide.
6) **Colour reagent B:** Stabilized chromogen (Tetramethylbenzidine).
7) **Stop solution:** 2 N sulfuric acid.
8) **Wash buffer:** 21 ml of a 25 fold concentrated solution of buffered surfactant with preservative.
9) **Substrate solution:** Colour reagent A and B were mixed in equal volumes in dark and to be used within 15 minutes.
10) **Total MMP-9 standard:** MMP-9 standard was reconstituted with 1 ml of D.D.W to produce a stock solution of 20 ng/ml.

Preparation of working standards:

- 500 µl of calibratior diluent was pipetted into each tube.
- 500 µl of stock solution was pipetted in first tube to produce working standard of 20 ng/ml

Assay Procedure

Prepared reagents, samples and standards as instructed.

⇩

Added 100 µl of assay diluent to each well.

⇩

Added 100 µl of standard, control or sample to each well and incubated 2 hours on the horizontal orbital microplate shaker at room temperature.

⇩

Aspirated and wash 4 times.

⇩

Added 200 µl of conjugate to each well and incubated 1 hour on the shaker at room temperature. Then aspirated and wash 4 times.

⇩

Added 200 µl of substrate solution to each well, protect from light and incubated half an hour on the bench top.

⇩

Added 50 µl of stopping solution to each well.

⇩

Read at 450 nm within half an hour (λ correction 540 or 570 nm) on ELISA plate reader.

TIMP-1 ELISA

Principle:

This assay is based on the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TIMP-1 has been pre-coated on a micro plate. Standards, controls and samples are pipetted into the wells, and any TIMP-1 present is bound by the immobilized antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for TIMP-1 is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour

develops in proportion to the amount of TIMP-1 bound in the initial step. The intensity of the colour is measured.

Sample preparation:

Serum samples were diluted 1: 50 using calibrator diluent provided in kit.

Reagent preparation:

All the reagents provided with kit were either lyophilized, concentrated or in ready to use form and were reconstituted according to the instruction manual.

1) **TIMP-1 micro plate:** 96 well polystyrene micro plate coated with a mouse monoclonal antibody against TIMP-1.
2) **TIMP-1 conjugate:** Polyclonal antibody against TIMP-1 conjugated to horseradish peroxidase, with preservatives.
3) **Assay diluent:** Buffered protein based with preservative.
4) **Calibrator diluent:** Buffered protein based with preservative.
5) **Colour reagent A:** Stabilized hydrogen peroxide.
6) **Colour reagent B:** Stabilized chromogen (Tetramethylbenzidine).
7) **Stop solution:** 2 N sulfuric acid.
8) **Wash buffer:** 21 ml of a 25 fold concentrated solution of buffered surfactant with preservatives.
9) **Substrate solution:** Colour reagent A and B were mixed in equal volumes in dark and to be used within 15 minutes.
10) **TIMP-1 standard:** TIMP-1 standards were reconstituted with 2 ml of D.D.W to produce a stock solution of 10 ng/ml.

Preparation of working standards:

- 200 µl of calibratior diluent was pipetted into each tube.
- Use the stock solution to produce a dilution series.
- The undiluted standard serves as higher concentration(10 ng/ml)

Assay Procedure

Prepared reagents, samples and standards as instructed.

⇩

Added 100 µl of assay diluent to each well.

⇩

Added 50 µl of standard, control or sample to each well and incubated 2 hours on the shaker at room temperature.

⇩

Aspirated and wash 3 times.

⇩

Added 200 µl of conjugate to each well and incubated 2 hours on the shaker at room temperature.

⇩

Aspirated and wash 3 times.

⇩

Added 200 µl of substrate solution to each well, protect from light and incubated half an hour on the bench top.

⇩

Added 50 µl of stopping solution to each well.

⇩

Read at 450 nm within half an hour (λ correction 540 or 570 nm) on ELISA plate reader.

TIMP-2 ELISA

Principle:

This assay is based on the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TIMP-2 has been pre-coated on a micro plate. Standards, controls and samples are pipetted into the wells, and any TIMP-2 present is bound by the immobilized antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for TIMP-2 is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of TIMP-2 bound in the initial step. The intensity of the colour is measured.

Sample preparation:

Serum samples were diluted 1: 50 using calibrator diluent provided in kit.

Reagent preparation:

All the reagents provided with kit were either lyophilized, concentrated or in ready to use form and were reconstituted according to the instruction manual.

1) **TIMP-2 micro plate:** 96 well polystyrene micro plate coated with a mouse monoclonal antibody against TIMP-2.
2) **TIMP-2 conjugate:** Polyclonal antibody against TIMP-2 conjugated to horseradish peroxidase, with preservatives.
3) **Assay diluent:** Buffered protein based with preservative.
4) **Calibrator diluent:** Buffered protein based with preservative.
5) **Colour reagent A:** Stabilized hydrogen peroxide.
6) **Colour reagent B:** Stabilized chromogen (Tetramethylbenzidine).
7) **Stop solution:** 2 N sulfuric acid.
8) **Wash buffer:** 21 ml of a 25 fold concentrated solution of buffered surfactant with preservatives.
9) **Substrate solution:** Colour reagent A and B were mixed in equal volumes in dark and to be used within 15 minutes.
10) **TIMP-2 standard:** TIMP-2 standards were reconstituted with 1 ml of D.D.W. to produce a stock solution of 100 ng/ml.

Preparation of working standards:

- 250 µl of calibratior diluent was pipetted into each tube.
- Use the stock solution to produce a dilution series. The standard serves as higher concentration (10 ng/ml)

Assay Procedure

Prepared reagents, samples and standards as instructed.

⇩

Added 100 µl of assay diluent to each well.

⇩

Added 50 µl of standard, control or sample to each well and incubated 2 hours on the shaker at room temperature. Then aspirated and washed 4 times.

⇩

.Added 200 µl of conjugate to each well and incubated 1 hour on the shaker at room temperature.

⇩

Aspirated and washed 4 times.

⇩

Added 200 µl of substrate solution to each well, protect from light and incubated half an hour on the bench top.

⇩

Added 50 µl of stopping solution to each well.

⇩

Read at 450 nm within half an hour (λ correction 540 or 570 nm) on ELISA plate reader.

Statistical analysis:

- The statistical significance of the data was determined using SPSS software [Version 10.0]. Following statistical methods were used for data analysis.
- Student `t' test was used to compare mean levels of the parameters between various groups of the subjects.
- ROC curve were constructed to determine the discriminating efficacy of the markers between various groups of the subjects.
- Pearson's correlation analysis was used to assess the association between markers in the subjects.
- Multivariate analysis was carried out to correlate the markers with clinicopathological parameters.
- Values of the parameters were expressed as Mean ± S.E.M. $P \leq 0.05$ were considered statistically significant.