Materials and Methods
Samples and Method

Chemicals

Protein-A agarose affinity column, methylglyoxal (MG), 1-anilinonaphthalene-8-sulphonic acid (ANS), guanidinium hydrochloride (Gn-HCl), sodium dodecyl sulphate (SDS), sodium azide, dialysis tubing, 5, 5′ – dithio-bis (2-nitro benzoic acid) (DTNB), thioflavin-T (ThT), congo red (CR), carboxyethyllysine (CEL), L-3,4-dihydroxyphenylalanine (L – DOPA), 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), freund’s complete adjuvant (FCA), freund’s incomplete adjuvant (FIA), bovine serum albumin (BSA), molecular weight markers, anti-rabbit and anti-human IgG alkaline phosphatase conjugate, para-nitrophenyl phosphate (PNPP), tween-20, hemoglobin (Hb), human serum albumin (HSA), H2A histone, arginine, lysine, phenyl alanine, tyrosine, tryptophan were purchased from Sigma Chemical Company, U.S.A. Acrylamide, bisacrylamide, ammonium persulphate (APS) and N,N,N′,N′–tetramethylethylenediamine (TEMED) were from Bio–Rad Laboratories, U.S.A. 2,4-Dinitrophenyl hydrazine (DNPH) and silver nitrate (AgNO3) were from SRL (India), sodium dihydrogen phosphate (NaH2PO4), sodium hydrogen phosphate (Na2HPO4), sodium chloride (NaCl), sodium carbonate (Na2CO3), sodium bicarbonate (NaHCO3), ethylenediaminetetraacetic acid (EDTA), methanol (CH3OH), ethanol (C2H5OH), ethyl acetate (C4H8O2), glycerol (C3H8O3), sodium thiosulphate (STS), sodium hydroxide (NaOH), hydrochloric acid (HCl), formaldehyde (HCHO), tris (hydroxymethyl) amino methane hydrochloride, trichloro acetic acid (TCA), trifluoro acetic acid (TFA), isopropanol, glycine, hydrogen peroxide (H2O2), ferrous chloride (FeCl2) were obtained from Qualigens, India. All other reagents and chemicals used in the study were of highest analytical grade available.

Equipments

The major equipments used in this study are Shimadzu UV-1700 spectrophotometer attached to a temperature programmer and controller unit (Japan), Shimadzu RF–5301 spectrofluorometer, Microcomputer equipped Jasco–J 815 spectropolarimeter, ELISA microplate reader (Labsystem Multiskan EX, Finland), ELICO, LI–120 pH meter (India), Polyacrylamide gel electrophoresis assembly (Genei, India), Kubota 6500 table top high speed refrigerated centrifuge (Osaka, Japan), Gel–Doc (Bio–Rad laboratories, USA), Fourier transform infrared (FTIR)
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spectrometer (Perkin Elmer, Germany), Scanning electron microscope (SEM) and Transmission electron microscope (TEM) with energy dispersive X-ray (JEOL, Japan), Dyna-pro-TC-04 dynamic light scattering equipment (Wyatt Technology, CA, USA), VP-DSC microcalorimeter (microcal, Northampton, MA), MALDI/MS – ABI Sciex 5800/ TOF system with LC- MALDI (AB SCIEX USA), LCMS – Waters SYNAPT G2 with 2D nano ACQUITY system (USA).

Collection of blood samples

This study had 80 patients with type 2 diabetes mellitus (T2DM) and 80 patients with rheumatoid arthritis (RA). Blood samples were collected from 80 T2DM patients aged more than 20 years, excluding those with micro and macro-vascular complications, type 1 diabetes mellitus (T1DM) and gestational diabetes mellitus (GDM). Twenty healthy subjects of the same age group form the control. Blood was taken in clot activator vials only after written informed consent from both the patients and healthy individuals. Patients with infection, known malignancy, pregnancy, cirrhosis, respiratory disorders, cardiovascular complications, alcohol consumption and smoking history were not included. Serum was separated by centrifugation at 3000 rpm for 10 min followed by heating at 56 °C for 30 min to inactivate complement proteins and samples were stored in aliquots at -20 °C with 0.1 % sodium azide preservative (Arafat et al., 2014). Similarly, blood was collected from RA patients satisfying the criteria of American Rheumatism Association. The patients (n = 80, mostly females) satisfied the criteria of American Rheumatism Association. The selected patients had no other autoimmune disease. Erythrocyte sedimentation rate (ESR) and level of C-reactive protein in serum (CRP) in RA sera was analysed by Western green method (Gambino et al., 1965) and nephelometric assay (Pearle et al., 2007) respectively. The isolated sera was stored at -20 °C with 0.1 % sodium azide as preservative until analysis.

Methods

Determination of protein concentration

Protein estimation was done by the method of Lowry et al., (1951) and Bradford (1976).
1. **Lowry method**

    Alkali (for keeping pH high), Cu$^{2+}$ ions (for protein chelation) and tartarate (for keeping Cu$^{2+}$ ions in the solution at high pH) are required for protein estimation by this method.

    **The reagents used in Lowry method are**

    a) Folin-Ciocalteau reagent.

    Commercially available reagent is diluted 4 times with distilled water prior to use.

    b) Alkaline copper reagent.

    It is made from 2 components (I and II) which are prepared in the following way:

    (I) Sodium carbonate (2 %) in 100 mM sodium hydroxide.

    (II) Copper sulphate (0.5 %) in 1 % sodium potassium tartarate

    Component (I) and (II) in a ratio of 1:50 is the working reagent, made fresh before use.

    **Procedure**

    1 ml of protein (varying concentrations) is added to 5.0 ml of freshly prepared alkaline copper reagent followed by a 10 min incubation. The incubated samples were then mixed with 1 ml of working Folin-Ciocalteau reagent and kept for 30 min at room temperature. A standard plot of BSA was constructed after taking absorbance at 660 nm. The plot is used to determine concentration of unknown sample.

2. **Bradford method**

    In this method, a shift in absorbance maxima from 465 to 595 nm of acidic solution of Coomassie Brilliant Blue G-250 is seen when protein binds to the dye. The anionic form of dye is then stabilised by hydrophobic and ionic interactions causing a visible colour change.
a) **Dye preparation**

It is prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 to 50 ml of 95 % ethanol followed by the addition of 85 % (v/v) orthophosphoric acid. The mixture is diluted to get a final volume of 1 litre. Undissolved particles are removed using whatman filter paper.

b) **Protein assay**

5 ml of dye is added to 1 ml of solution containing 10 to 100 µg of protein followed by vortexing. After 5 min, absorbance was taken at 595 nm against blank reagent.

**IgG Isolation**

Blood samples from healthy individuals were obtained and allowed to coagulate at 37 ºC for 30 min. It was followed by its centrifugation at 3000 rpm for 10 min to obtain the serum which was heated at 56 ºC for 30 min to inactivate complement proteins. IgG was isolated by affinity chromatography using Protein A-agarose column and its concentration and purity was determined as per the established protocol (Arfat et al., 2016). The homogeneity of isolated IgG was checked on 7.5 % SDS-PAGE and it was stored at -20 °C with 0.1 % sodium azide as preservative.

**Hydroxyl radical (OH•) generation and IgG modification**

Hydroxyl radical (OH•) was generated by Fenton’s reaction which involves reaction between Fe^{2+} and H_{2}O_{2} producing OH•. IgG modification was carried out by incubating 1 µM of IgG dissolved in 10 mM PBS (pH 7.4) with 100 µM H_{2}O_{2} and increasing concentrations of FeCl_{2}/EDTA (10 µM, 20 µM and 30 µM) for half an hour at 37 ºC. The reaction was then stopped using 0.1 % TFA. There was no marked increase in percent hyperchromicity with further increase in FeCl_{2} concentration. EDTA chelates Fe^{3+} generated in Fenton’s reaction. Two parallel reactions having IgG alone in PBS and IgG with 100 µM H_{2}O_{2} served as control. After incubation, the solutions were extensively dialyzed in PBS to remove excess H_{2}O_{2} (Alam et al., 1993; Guedes et al., 2011).
IgG modification by Methylglyoxal (MG)

IgG modification was carried out by incubating 1 µM of IgG dissolved in 10 mM PBS (pH 7.4) with increasing concentrations of MG (1.5 mM, 3 mM, 4.5 mM, 6.0 mM and 7.5 mM) for 24 hours at 37 °C. There was no marked increase in percent hyperchromicity with further increase in MG concentration. After incubation, the solutions were extensively dialyzed in PBS to remove unbound MG (Mir et al., 2014).

Preparation of hydroxyl radical (OH•) modified MG glycated IgG

IgG (1 µM) was incubated under sterile conditions with MG (7.5 mM) in phosphate buffer saline (10 mM, pH 7.4) at 37 °C in capped sterile tubes for 24 hours. It was followed by OH• modification of the glycated samples. OH• was generated by Fenton’s reaction. Assay tubes containing glycated samples i.e. MG modified IgG (MG-IgG) were incubated with 100 µM H₂O₂ and 30 µM FeCl₂/EDTA for half an hour at 37 °C. The reaction was then stopped by using 0.1 % TFA (Guedes et al., 2011). The cumulative effect of OH• and MG on IgG i.e. OH• treated MG glycated IgG was studied with native IgG (N-IgG), MG modified IgG (MG-IgG) and OH• modified IgG (OH•-IgG) as control samples. The samples were extensively dialyzed against sterile PBS buffer (10 mM, pH 7.4) to remove excess reagent and stored at -20 °C until further analysis.

Physicochemical characterisation of N-IgG, OH•-IgG, MG-IgG and OH•-MG-IgG

UV Absorbance spectroscopy

The absorption profile of N-IgG, OH•-IgG (varying concentration), MG-IgG (varying concentration) and OH•-MG-IgG (with chosen concentration of MG and OH•) were recorded in Shimadzu spectrophotometer (UV 1700 model) in the wavelength range of 250-400 nm using quartz cuvette of 1 cm path length (Allarakha et al., 2015). Percent increase in hyperchromicity was evaluated by the following equation:

\[
\text{% increase in hyperchromicity} = \frac{\text{OD}_{\text{modified IgG}} - \text{OD}_{\text{native IgG}}}{\text{OD}_{\text{modified IgG}}} \times 100
\]
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**Fluorescence intensity measurements**

Fluorescence spectra of N-IgG, OH⁻-IgG, MG-IgG and OH⁻-MG-IgG was recorded on Shimazdu (RF-5301-PC) spectrofluorometer at 25 °C in a quartz cuvette of 1 cm path length. Tyrosine and tryptophan fluorescence intensities were monitored by exciting N-IgG, OH⁻-IgG, MG-IgG and OH⁻-MG-IgG at 275 nm and 295 nm and emission spectra were recorded in the range of 280-400 nm and 300-400 nm respectively (Ahmad et al., 2012). Loss in the fluorescence intensity (FI) was calculated using following equation:

\[
\% \text{ loss in fluorescence intensity} = \frac{\text{FI}_{\text{native IgG}} - \text{FI}_{\text{modified IgG}}}{\text{FI}_{\text{native IgG}}} \times 100
\]

**Gel electrophoresis**

SDS–PAGE of N-IgG, OH⁻-IgG, MG-IgG and OH⁻-MG-IgG was performed as per a published protocol (Laemmli., 1970). The following solutions were prepared

*Acrylamide – Bisacrylamide (30: 0.8)*

The solution was prepared by mixing 30 gm of acrylamide and 0.8 gm of bisacrylamide. The volume was made up to 100 ml with distilled water. The solution was filtered and stored in dark bottle at 4 °C.

**Resolving buffer**

It was prepared by dissolving 9.08 gm of 1.5 M tris in 40 ml distilled water. The pH of the solution was then adjusted to 8.8 using 6 N HCl. The final volume was adjusted to 50 ml.

**Running/ Electrophoresis buffer**

3 gm of 0.025 M tris, 14.4 gm of 0.2 M glycine and 1 gm of SDS is dissolved in distilled water. Final volume was made upto 1.0 litre with distilled water.

**Casting of polyacrylamide gel**

Washed and ethanol cleaned glass plates were separated with 1.5 mm thick spacers and casted on a stand. The plates were sealed from sides and bottom with 1 % agarose. Resolving gel mixture (6 %) was prepared by adding the components given
in table 3. The mixture was then poured between the glass plates and allowed to polymerise at room temperature. 20 µl of 3 µg/ml of IgG samples (N-IgG, OH’-IgG, MG-IgG and OH’-MG-IgG) is mixed with 5 µl of sample dye. The mixture was heated and loaded onto the wells. Electrophoresis was performed at 80 V for 4 hrs at room temperature and the protein bands in the gel were visualized by silver staining. The Gel was photographed using Molecular Imager Gel Doc XR system (Laemmli., 1970).

Table-3

Components used for preparing resolving gel mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (10 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>2 ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5.3 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.008 ml</td>
</tr>
</tbody>
</table>

Circular Dichroism (CD)

Far and near-UV CD measurements were carried out on a Jasco spectropolarimeter (J-815) equipped with a Jasco Peltier type temperature controller (PTC-424s/15) in the wavelength range of 250-190 nm and 320-250 nm respectively. The instrument was calibrated with D-10- camphor sulphonic acid. Spectra were taken in a cell of 1 and 10 mm path length. The CD shape constant (S value) was taken from the following equation (Chen et al., 1974; Li et al., 2005).

\[ S\text{ value} = \frac{\text{Ellipticity at 200 nm}}{\text{Ellipticity at 217 nm}} \]
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**Fourier transform infrared spectroscopic analysis (FTIR)**

FTIR spectroscopy of the protein samples (5 mg/ml) was performed on Perkin Elmer FTIR spectrophotometer in the wave number range between 1500 cm\(^{-1}\) - 1700 cm\(^{-1}\). Sample concentration was 5 mg/ml (Mir et al., 2014).

**Effective protein hydrophobicity**

Binding of ANS to N-IgG, OH\(^{-}\)-IgG, MG-IgG and OH\(^{-}\)-MG-IgG was evaluated using fluorescence measurements. The molar ratio of protein to ANS was 1:50 and emission spectra were recorded in the wavelength range of 400-600 nm after exciting the samples at 380 nm (Amani et al., 2011). Increase in fluorescence intensity was calculated as given below:

\[
\text{% increase in fluorescence intensity} = \frac{\text{FI}_{\text{modified IgG}} - \text{FI}_{\text{native IgG}}}{\text{FI}_{\text{modified IgG}}} \times 100
\]

**Determination of reactive carbonyl content**

Carbonyl content of native and modified IgG was determined according to a published protocol (Hawkins et al., 2009). 250 µl of 10 mM DNPH in 2.5 M HCl was added to equal volume of IgG (5 mg/ml). The mixture was incubated in dark for 15 min at room temperature with regular vortexing at every 5 minutes. Thereafter, 125 µl of TCA solution was added followed by incubation at -20 ºC for 15 min. The mixture was centrifuged at 4 ºC for 15 min at 9000 g. Supernatent was discarded and pellet was washed thrice with ice cold ethanol/ethyl acetate. Absorbance was taken at 370 nm after redissolving pellet in guanidinium hydrochloride (GuHCl). The carbonyl content in native and modified samples was calculated using extinction coefficient of 22,000 M\(^{-1}\) cm\(^{-1}\). Protein carbonyl content is expressed in nmole/mg IgG.

**Determination of free sulphydryl group content**

Sulphydryl group content of N-IgG, OH\(^{-}\)-IgG, MG-IgG and OH\(^{-}\)-MG- IgG was calculated according to Ellmans method (Sedlak et al., 1968). Native and modified IgG (5 mg/ml) was mixed with 3000 µl DTNB. The mixture was incubated at room temperature for 30 min in dark. The absorbance was taken at 412 nm against DTNB serving as blank. Thiol content of native and modified samples was calculated
using extinction coefficient $13600 \text{ M}^{-1} \text{ cm}^{-1}$. Protein thiol content is expressed in n mole/mg IgG.

**Dynamic light scattering (DLS)**

DLS measurement of N-IgG, OH’-IgG, MG-IgG and OH’-MG-IgG were carried out at 830 nm using Dyna-pro-TC-04 dynamic light scattering equipment (Wyatt Technology, CA, USA). All the solutions were spun at 10,000 rpm for 10 min followed by filtration through a microfilter (whatman International, maidstone, UK). The protein concentration was taken at optimum resolution using Dynamics 6.10.0.10 software as reported earlier (Rabbani et al., 2012).

**Differential scanning calorimetry (DSC)**

DSC was performed on VP-DSC microcalorimeter (microcal, Northampton, MA). Scans were run in the range of 20-90 °C at the rate of 1 °C/min using 3 mg/ml of N-IgG, OH’-IgG, MG-IgG and OH’-MG-IgG. The heat capacity ($C_p$), thermal denaturation temperature ($T_m$) and enthalpy change ($\Delta H$) were analyzed by using 7.0 scientific plotting software (Ionescu et al., 2008).

**Thioflavin T (ThT) and Congo Red (CR) binding studies**

Modification induced aggregation and cross linking was evaluated by ThT and CR binding assays. N-IgG, OH’-IgG, MG-IgG and OH’-MG-IgG samples in a 1:2 molar ratio were incubated with ThT (for 1 hr) and with CR (for 30 min) respectively. Protein samples incubated with ThT were analysed on Shimazdu (RF-5301-PC) spectrofluorometer at 25 °C in a quartz cuvette of 1 cm path length. Spectral analysis of protein samples incubated with CR was done in 300-700 nm range for absorbance analysis for further confirmation of aggregate formation (Takashima., 2001).

**Spectacle crosslink’s in modified IgG**

CR staining was done to study aggregation as a result of protein (IgG) modification. Staining was done as per previously published procedure (Lorenzo and Yankner., 1994). Briefly, 200 – 400 µl of freshly prepared CR solution was put on slides having 10 µl of air dried IgG (5 µM). The slides were then examined under polarised light microscope. The detection of yellow or green colour birefringence
indicates the presence of the protein aggregates in the samples. Images of the stained slides were recorded with the camera attached to the microscope.

**Scanning electron microscopy (SEM)**

Scanning electron microscope (SEM) imaging was used to analyze changes in microarchitectural of the protein as a result of modification. 20 μl of 5 μM sample was air-dried and then adsorbed on cellulose ultra-filtration membrane. Further processing involved coating the samples with gold and mounting them on carbon tape coated stainless steel grids operating under low vacuum at 15 kV. Scanning electron micrographs were recorded at 1000X magnification on a JSM-6510LV (JEOL JAPAN) (Iram and Naeem, 2013).

**Transmission electron microscopy (TEM)**

TEM studies of native and modified IgG (5 μM) were performed on JEOL transmission electron microscope operated at an accelerating voltage of 180 kV. Sample preparation was done by putting 5 μM protein on carbon stabilised forvar film adsorbed copper grid stained negative with 2 % of heavy metal salt, uranyl acetate. Images were viewed at 15,000X on a computer screen when electrons emitted from tungsten coated filament of electron gun operating at a high negative voltage were allowed to focus on sample through condenser lens (Khan et al. 2012).

**RBC hemolysis assay**

The antioxidant characteristics of native and modified IgG were evaluated by RBC haemolysis assay (Stocker et al., 2003). Blood samples were collected from healthy human subjects in EDTA vials. Erythrocytes from the plasma were isolated and washed three times with isotonic saline (NaCl, 0.15 M), centrifuged at 4000 rpm for 10 minutes at 4 °C. 100 μl (approx 1 X 10^8 erythrocytes, 400,000 cells/μl final concentrations) of diluted red blood cells (1/10 in 0.15 M NaCl) were added into each well of flat bottom 96 well-plate. 10 μM each of N-IgG, OH⁻-IgG, MG-IgG and OH⁻-MG-IgG were then added to the well in duplicate. This was followed by addition of 20 μl of 200 mM of 2', 2'- AAPH, a peroxyl radical initiator. Diluted RBC alone and RBC with AAPH were taken as positive and negative controls respectively. The samples were incubated at 37 °C and absorbance was recorded on microplate reader at 540 nm every 20 min until constant readings were achieved in each well (Ahmad et
al., 2015). Results are expressed as time (in minutes) required for 50 % of maximal inhibition in haemolysis of RBCs (HT 50) using the following equation:

\[ \% \text{ inhibition} = \frac{[A_{\text{AAPH}} - A_{\text{sample}}]}{A_{\text{AAPH}}} \]

\(A_{\text{AAPH}}\) = Absorbance of AAPH at 540 nm

\(A_{\text{sample}}\) is the absorbance of IgG (native and modified) at 540 nm.

Matrix-assisted laser desorption ionisation-mass spectrometry (MALDI-MS) analysis

MALDI analysis was performed on 4800 plus MALDI-TOF/TOF mass spectrometer operating in a positive ion mode. 0.95 µl of 1 mg/ml sample was spotted on a 384 well insert opti-TOF-stainless steel MALDI plate. Sinapinic acid dissolved in 10 mg/ml acetonitrile and 0.1 % TFA was used as a matrix. Analysis was carried out using protein chip software.

Immunisation schedule

Immunisation of randomly bred female New Zealand white rabbits was done as per the published protocol (Dixit et al., 2005). Preimmune blood was collected from marginal ear vein of rabbits (n = 8, 2 each for N-IgG, OH’-IgG, MG-IgG and OH’-MG-IgG). Serum was separated from blood samples, complement proteins were heat inactivated and it was stored at -20 ºC with 0.1 % of sodium azide as preservative. Rabbits were immunised intramuscularly at multiple sites with 100 µl of 10 µg/ml of the respective antigen (native and modified counterparts) emulsified with equal volume of complete freund’s adjuvant. This was followed by 4 booster doses, each given at an interval of one week in incomplete freund’s adjuvant. Blood was collected from marginal ear vein one week post immunisation, sera was separated and processed similarly as preimmune. The study protocol was approved by institutional animal ethical committee.

Hematological and biochemical parameters

Hematological parameters (White blood cells, Red blood cells, Neutrophils, Eosinophils, Basophils, Platelets) were determined on preimmune and immune blood of rabbits collected in K3-EDTA anticoagulant vials using Sysmex hematological automated analyzer (Sysmex Corporation Lincolnshire, IL 60069).
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Rectal temperature measurement

The rectal temperature of rabbits treated with N-IgG and OH⁻-MG-IgG was recorded with high capacity sensitive digital thermometer at the termination (6th week post immunization) of experiment.

IgG isolation by affinity chromatography

IgG from human and animal sera was isolated on protein-A agarose affinity column. Sera (0.5 ml) mixed with equal volume of PBS (pH 7.4) was applied to column after equilibration with the same buffer. The flow through samples were recycled 2-3 times, so that maximum IgG can get attached to Protein-A. Bound IgG was then eluted using a solution of 0.58 % acetic acid and 0.85 % NaCl. 3 ml fractions were collected in test tubes containing 1 ml of tris-HCl to neutralise IgG. Absorbance (OD) of the fractions were taken at 251 nm and 278 nm and the concentration of IgG was determined considering 1.4 OD<sub>278</sub> = 1 mg/ml IgG. This was followed by extensive dialysis against PBS and the samples were stored at -20 ºC for further use (Dixit et al., 2011).

Immunological detection of antibodies

Antibody in preimmune sera, immune sera, diabetes sera and RA sera were detected by Enzyme linked immunosorbent assay (ELISA) and band shift assay.

Enzyme linked immunosorbent assay (ELISA)

Following reagents were prepared for ELISA

Antigen coating buffer : Prepared by mixing 15 mM sodium carbonate with 35 mM sodium bicarbonate and adjusting the pH to 9.6.

Tris buffer saline (TBS): 10 mM Tris added to 150 mM NaCl and the pH was adjusted to 7.4.

Tris buffered saline containing Tween 20 (TBS-T): 20 mM Tris, 144 mM NaCl, 2.68 mM KCl, pH 7.4 and containing 500 µl Tween 20 per litre.

Carbonate-bicarbonate buffer: 15 mM sodium carbonate, 35 mM sodium bicarbonate, 2 mM MgCl₂, pH 9.6.
**Substrate:** 500 µg PNPP in 1 ml of carbonate – bicarbonate buffer.

**Procedure for ELISA**

It was done according to a published protocol (Ali and Alam., 2002) with slight modifications. Microtiter wells of 96 well polystyrene plates were respectively coated, in duplicate, with 100 µl of 10 µg/ml of N-IgG, OH-IgG, MG-IgG and OH-MG-IgG for 2 hours at 37 ºC and overnight at 4 ºC. Half of the plates devoid of antigen served as control. After incubation, the plates were washed thrice with TBS-T, to remove unbound antigen. This was followed by blocking of unoccupied sites with 2.5 % of fat free milk dissolved in TBS for 4-6 hours at 37 ºC. The plates were washed again, thrice with TBS-T. 100 µl of serially diluted test serum/ affinity purified IgG was poured into each well. The plates were kept aside at 37 ºC for 2 hours and 4 ºC overnight. Next day, the plates were washed 3–4 times with TBS-T and the bound antibodies were assayed by anti-rabbit/ anti-human alkaline phosphatase conjugate followed by a 2 hour incubation at 37 ºC, three times washing with TBS-T and then with distilled water. The colour was developed using para-nitrophenyl phosphate (PNPP) as substrate. Absorbance was measured at 410 nm and the results calculated as $A_{test} - A_{control}$.

**Competition ELISA**

Antigenic specificity of bound antibodies was determined by competition ELISA (Habib et al., 2005). This is similar to direct binding ELISA except that immune complex instead of serum/IgG is added to wells. Immune complex is prepared by incubating a mixture of varying concentration of inhibitor (0 – 20 µg/ml) with a constant amount of serum/IgG for 2 hours at room temperature and overnight at 4 ºC. Percent inhibition is calculated using the formula

$$\% \text{ inhibition} = 1 - \left( \frac{A_{inhibited}}{A_{uninhibited}} \right) \times 100$$

**Band shift assay**

This assay was done to visualise antigen-antibody binding and formation of immune complexes (Ansari et al., 2009). The complexes, prepared by incubating constant amount of native/ modified antigen with varying concentrations of affinity purified IgG in phosphate buffer saline at 37 ºC for 2 hours and 4 ºC overnight were
mixed with sample dye in 4:1 ratio and loaded onto wells of 6% SDS–polyacrylamide gel for 3 hours at an operating voltage of 80 V. Silver stain was used to visualise the band pattern.

**Statistical analysis**

Statistical evaluation of the results was carried out by Student’s t-test using Origin Software 6.1 (USA). Data are expressed as mean ± SD and a p value of <0.05 has been considered to be statistically significant.