

Development of monoclonal antibodies against chicken IgM and its application in immunity studies

S Rathnapraba*, G Dhinakar Raj and V Thiagarajan

Department of Animal Biotechnology, Madras Veterinary College, Tamilnadu Veterinary and Animal Sciences University
Chennai 600 007, India

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Immunoglobulin M (IgM) from specific pathogen free (SPF) chicken serum was purified and used for production of monoclonal antibodies (MAbs) for subsequent application in the detection of primary immune response to infectious agents. Chicken IgM was fractionated by gel filtration technique using Sephadex G-200. Purity of the fractionated IgM was confirmed by agar gel precipitation test (AGPT) and immunoelectrophoresis (IE) using rabbit anti-chicken serum. In polyacrylamide gel electrophoresis (PAGE), under non-reducing conditions, a single band was noticed and under reduced conditions, a band with molecular weight of 74 kDa was observed. Cell fusion was carried out with splenocytes from immunized BALB/c mice and Sp2/0 cells, and the high positive clones were selected and characterized for isotype and cross-reactivity with IgY in enzyme linked immunosorbent assay (ELISA). Three MAbs chosen for characterization were of IgG1 isotype and they did not cross-react with standard IgY in ELISA. Concentrated IgM MAbs developed in this study were used in ELISA for the measurement of immune response in sequentially collected serum samples of birds, experimentally infected with egg drop syndrome (EDS-76) virus. The purified virus was coated onto the ELISA plates, followed by the addition of sera samples to be tested, MAbs against chicken IgM, anti-mouse peroxidase conjugate and substrate. The assay revealed an increase in IgM response in individual birds from 5-12 d with a peak on 9th d post-inoculation (PI), followed by an increase in IgG on 12-25 d PI. IgM antibodies against infectious bursal disease (IBD) virus were tested in 34 field serum samples. Comparison with AGPT revealed a marginal increase in sensitivity with IgM ELISA for detection of IBD virus specific IgM.

Key words: Chicken IgM, ELISA, immunoglobulins, monoclonal antibodies

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Introduction

A plethora of viral diseases affecting chickens, which result in mortality and production losses, are the major constraints in development of poultry industry. Viral diseases are diagnosed either by isolation and identification of the causative virus, or demonstration of rising antibody titres to the causative virus. Enzyme linked immunosorbent assay (ELISA) is the most commonly used test and a wide variety of kits have been developed for assessment of sero-conversion. However, all the ELISA based kits use anti-chicken IgG conjugates that may cross-react with other classes of immunoglobulins (Ig), namely IgM and IgA, through binding to cross-reacting epitopes present either on the light or heavy chains.

The three most abundant classes of mammalian immunoglobulins, viz., IgG, IgM and IgA, each have

their equivalents in the chicken. Chicken IgM isolated from serum has a high molecular weight (900 kDa) and upon reduction dissociates into heavy chains 80 kDa and light chains 22 kDa¹. Chicken IgM is the first antibody observed after primary immunization and IgM is expressed on the surface of most chicken B cells. During the course of an immune response, IgM is produced during the first week post-infection with peak titres around d 5 post-inoculation (PI). During this time class switching occurs, resulting in a build up of IgG class of antibodies also. Detection of antigen specific IgM indicates incidence of a disease, while IgG responses indicates prevalence of a disease. The assay of antigen-specific IgM requires the availability of IgM-specific MAbs/serum.

Immunoglobulin class-specific MAbs can be developed through hybridoma technology. Chicken IgM MAbs, if available, can be used extensively in sero diagnosis of recent viral, bacterial and parasitic infections in poultry directly from serum samples. A sensitive ELISA for detecting infectious bronchitis

*Author for correspondence:
Tel: 91-442-25381506 extn ABT; Fax: 91-442-25389445
E-mail: drrathubiotech_2004@yahoo.co.in

virus (IBV) specific IgM antibodies after its separation from serum by gel filtration chromatography has been developed, which makes it less suitable for routine testing of large number of samples^{2,3}. For this assay, purified IBV was coated on to ELISA plates, followed by the addition of serum to be tested, Mabs against chicken IgM, anti-mouse peroxidase conjugate and substrate. Thus, only IBV-specific IgM responses were estimated. Separation of IgM from IgG in the serum was ensured by gel filtration chromatography to remove inter-class competition of antibodies against IBV. An IgM capture ELISA developed by Westernbrink and Kimman⁴ proved to be sensitive enough to detect IgM levels in experimentally/naturally infected animals. An antibody capture ELISA was described for the detection of IBV specific IgM response⁵. All these ELISA formats utilized IgM specific MAb either as detection or as capture antibody.

The objectives of the present study were three-fold: i) Isolation and purification of chicken IgM using chromatographic techniques; ii) Production and characterization of MAb against chicken IgM; and iii) Application of IgM MAb produced in diagnosis of recent infections in poultry.

Materials and Methods

Fractionation of IgM by Chromatographic Method

SPF eggs purchased from Western Hatcheries, Pune were hatched out at Poultry Research Station, Nandanam. The chicks were maintained for 2 wk at the Department of Animal Biotechnology under sterile conditions and fed and watered *ad libitum*. Serum pools obtained from them were centrifuged and the γ -globulin fraction was precipitated by sodium sulphate at a final concentration of 18% (w/v). Insoluble immunoglobulin pellet was resuspended and dialysed against phosphate buffered saline (PBS). The sodium sulphate precipitated pooled SPF chicken sera were fractionated by gel filtration chromatography, using the chromatography unit (Bio-Rad) in a 100 × 2.6 cm glass column packed with Sephadex G-200 (Amersham, USA) and eluted with PBS. The first peak containing chicken serum IgM fractions were pooled and the optical density (OD) was determined at 280 nm in a spectrophotometer. The protein concentration in mg/mL was obtained by multiplying OD value with a factor, 0.74 as described by Hudson and Hay⁶. The eluted fractions were tested for purity

and identity of IgM by agar gel precipitation test (AGPT) or immunoelectrophoresis (IE) against anti-chicken whole serum raised in rabbits and in SDS-PAGE following conventional procedures.

Production and Characterization of MAb Against Chicken IgM

A batch of 3 female BALB/c mice, 6-8-wk-old, obtained from National Institute of Nutrition, Hyderabad, were immunized with the fractionated chicken IgM with some modifications⁷. About 100 μ g of IgM antigen emulsified with 100 μ L Freund's complete antigen (FCA; Sigma, USA) was given intraperitoneally. Five booster injections were given with 80 μ g of chicken IgM antigen with 80 μ L of Freund's incomplete antigen (FIA) at 15 d interval. Finally, intravenous booster injections were given daily with 25 μ g of antigen dissolved in PBS for 3 d prior to cell fusion. Fusion was carried out following the standard procedure of Galfre and Milstien⁸. When the hybridomas attained 50% confluency, the culture supernatants were collected and tested for the presence of antibodies to IgM in an indirect ELISA⁹, using the following procedure. ELISA plates were coated with antigen (0.125 μ g/well of purified chicken IgM) diluted in coating buffer (100 μ L/well) and kept for overnight incubation at 4°C. The volume was kept constant in all other steps. The following day, the plates were washed with PBS Tween-20 (PBST) and unbound sites were blocked with 2% BSA by incubating at 37°C for 1 h. After washing, the dilutions of mouse anti-serum and the supernatants were added in duplicate and incubated at 37°C for 2 h. Then goat anti-mouse IgG HRP conjugate in PBS at 1:2000 dilution was added, incubated for 1 h and 2'-2' azino-diethyl benzthiazoline 6-sulphonic acid (ABTS) added. The colour developed was read at 405 nm. A positive control (hyperimmune serum of mouse) and a negative control (myeloma cell line Sp₂/0 supernatant) were included along with conjugate and substrate controls. An optical density value more than twice of the negative control was taken as positive (P/N \geq 2.0). Hybridoma secreting antibodies against IgM were cloned by limiting dilution technique thrice as per the procedures described by Goding¹⁰.

MAb against chicken IgM were further characterized for their isotype and specificity. Class and subclass of MAb produced were determined by sandwich ELISA using a mouse monoclonal antibody isotyping kit (Sigma Chemical Company, USA).

Isotype specific mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA Mabs were used as coating antigen. Following washing and addition of positive culture supernatants, peroxidase labeled goat anti-mouse IgG (F_{ab} specific) conjugate was added for detection. The substrate used was ABTS and the OD read at 405 nm.

The specificity of chicken IgM MABs was confirmed by analyzing its cross-reactivity with purified standard chicken IgY (Promega, USA) using an indirect ELISA. Briefly, fractionated IgM (0.125 µg/well) and the standard IgY (0.125 µg/well) were used as coating antigens in ELISA against supernatants from the clones secreting IgM MABs. Goat anti-mouse HRP conjugate was used, followed by the addition of substrate ABTS. The colour development was read at 405 nm.

Study of Immune Response in Birds Experimentally Infected with EDS 76 Virus

Ten 5-wk-old chickens obtained from Poultry Research Station, Nandanam were tagged individually and housed in clean isolated conditions. A local EDS virus strain TN4¹¹, available in the Department of Animal Biotechnology, was inoculated at the rate of 0.1 mL of infective allantoic fluid per bird by the oral route so that the birds received log 10^{6.0} 50% egg infective doses (EID₅₀). These infected birds were regularly bled sequentially between 0 and 25 d after inoculation. Serum was separated and stored at -20°C. Serum from 4 uninoculated chicks was used as negative control serum. ELISA for detection of IgM antibodies in chicken serum was performed as per the method of Da Silva Martins *et al.*³ using MABs concentrated by ultrafiltration with a cut off membrane of 100 kDa (Jumbosep, Pall Gelman, USA). Briefly, ELISA plates were coated with EDS antigen (2 µg/well) and incubated at 4°C overnight. Test sera (1:500 dilution) collected from EDS infected birds on different days from d 0 to d 25 PI were added and incubated for 1 h at 37°C. After washing with PBST, anti-IgM MABs developed were added and incubated at 37°C for 2 h. Subsequent to washing, anti-mouse IgG HRP conjugate at 1:2000 dilution was added and the colour development was detected by the addition of substrate ABTS. The stopping solution used was 5% SDS, and the OD read at 405 nm. The mean OD values of the uninoculated EDS-negative control sera in IgM ELISA was calculated and serum samples showing OD greater than mean±3 S.D. were taken as positive for the presence of EDS virus-specific IgM.

To assess the antigen-specific IgG responses, a conventional indirect ELISA was done with the same antigen and test sera, followed by addition of anti-chicken IgY peroxidase conjugate at 1:1000 dilution, detected by ABTS and OD values read. Appropriate positive control serum, negative control serum, Sp2/0, HRP and substrate control were included in each test.

Detection of IgM Response to IBD Virus in Field Serum Samples

Thirty four serum samples obtained from a breeder flock from Thuraiyur, Tamil Nadu were subjected to antigen-specific indirect IgM ELISA as well as in AGPT for detection of antibodies to IBD virus following the procedure of De Witt *et al.*⁵. IBD antigen-specific IgG responses were also estimated as before for EDS virus using a conventional indirect ELISA and anti chicken IgY peroxidase conjugate. Appropriate positive control serum, negative control serum, Sp2/0, HRP and substrate control were included in each test.

Results

Fractionation of Chicken IgM by Chromatographic Method

Elution pattern of the sodium sulphate precipitated SPF chicken sera in Sephadex G-200 resulted in two peaks (Fig. 1). The first peak fractions that contained IgM were pooled and the concentration was estimated to be 0.85 mg/mL. A single precipitation line appeared between IgM rich fractions (1st peak) and anti-chicken serum, both in the AGPT (Fig. 2a) and IE tests (Fig. 2b). In SDS-PAGE, under non-reducing conditions, a band above the IgG band was seen, while under reducing conditions, a band with molecular weight of 74 kDa was noticed in the IgM fraction (Fig. 3). In the standard IgY and the pooled fractions of the second peak, a heavy chain band with the molecular weight of 58 kDa was noticed. The light chain band was not clearly visible.

Production and Characterization of MABs Against Chicken IgM

Cell fusions were carried out using splenocytes from high titred mice at 80 and 100 d of immunization. A total of 225 hybridomas were obtained with a fusion efficiency of 68.18 per cent. Following sub cloning of three positive hybridoma clones thrice, six clones (3H₁₀B₂, 3H₁₀G₁₀, 3H₁₀B₆, C₃B₅, C₃D₁₂ and C₃D₈) were chosen based on high OD values obtained in an indirect ELISA at 405 nm with IgM as the coating antigen and used for

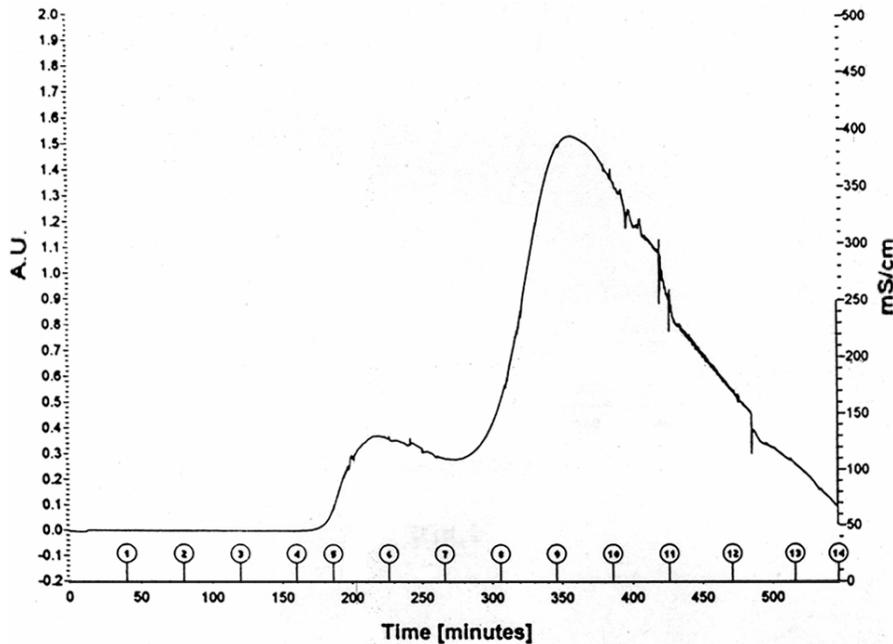


Fig. 1—Elution profile of Na_2SO_4 precipitated SPF chicken serum passed through sephadex G-200, (first peak rich in IgM and second peak IgG)

isotyping. All these clones were found to belong to the IgG1 subclass. None of these clones showed cross-reactivity to chicken IgY in ELISA (Table 1).

Study of Immune Response in Birds Experimentally Infected with EDS 76 Virus

The antigen-specific IgM and IgG responses of chickens following a single oral inoculation with EDS₇₆ virus was studied in an indirect ELISA with the IgM MAbs developed. The IgM antibodies reached a maximum level between 5-12 d PI in all the experimentally infected chickens and then declined. IgY antibodies to EDS virus was less during 5-12 d PI and then started increasing from d 12 PI (Fig. 4). Minor variations were observed between individual birds with respect to peak antigen-specific IgM titres obtained.

Detection of IgM Response to IBD Virus in Field Sera Samples

The mean OD values of the known IBD-negative sera in IgM ELISA was found to be 0.13 and serum samples showing OD greater than mean \pm 3 S.D., i.e. samples with a OD above 0.39, were taken as positive for the presence of IBD virus-specific IgM. Using this cut off, 24 out of 34 (70.6%) samples were found positive for the presence of IgM antibodies while only 61.8% (21 out of 34) were found positive in AGPT. Thus, IgM ELISA detected 3 samples as positive, which were found to be negative by AGPT.

Table 1—Cross-reactivity of IgM Mabs against chicken IgY in ELISA

No.	Clones	OD values	
		Chicken IgM	Chicken IgY
1	H ₁₀ E ₂	1.621	0.175
2	*H ₁₀ G ₁₀	1.730	0.189
3	H ₁₀ D ₄	1.512	0.197
4	*H ₁₀ B ₂	1.910	0.195
5	*H ₁₀ B ₆	1.501	0.199
6	*C ₃ D ₈	1.756	0.190
7	*C ₃ B ₅	1.715	0.201
8	*C ₃ D ₁₂	1.836	0.211
9	C ₃ D ₂	1.647	0.199

None of these clones showed cross-reactivity to chicken IgY.

*The six clones (H₁₀G₁₀, H₁₀B₂, H₁₀B₆, C₃D₈, C₃B₅ and C₃D₁₂) were chosen based on high OD values obtained in ELISA.

Discussion

Elution profile of Na_2SO_4 precipitated chicken γ -globulins through the Sephadex G-200 gel filtration consisted of two peaks, the first peak rich in IgM and second peak in IgG. IgM, being the heavier immunoglobulin, was eluted in the first peak. This elution pattern of IgM, IgG obtained in this study was similar to that of previously described by Goel *et al*¹². The concentration of IgM was 0.85 mg/mL. Separation of IgM from fowl serum by Na_2SO_4

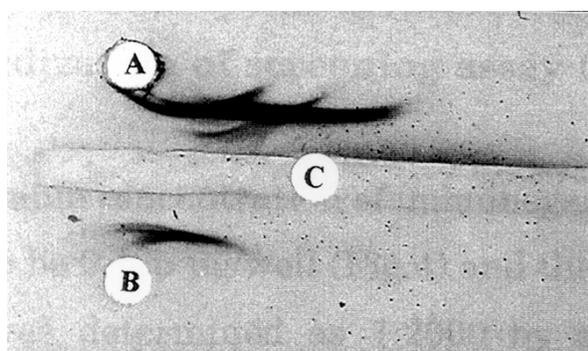
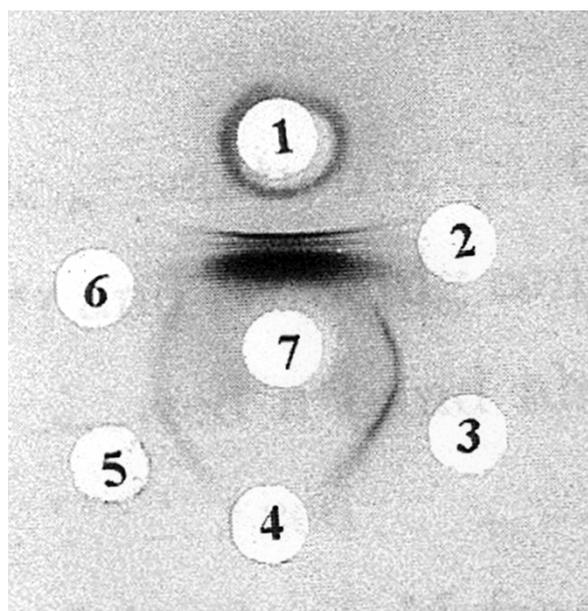


Fig. 2—Immuno diffusion and immuno electrophoretic patterns of IgM preparations and rabbit anti-chicken sera—A. Immuno diffusion: 1, Chicken serum; 2-6, 1st peak fractions of Sephadex G-200; 4, Negative control; and 7, Anti-chicken sera; B. Immuno electrophoresis: a, Chicken sera; b, First peak fraction of Sephadex G-200; and c, Rabbit anti-chicken sera in trough.

precipitation and gel filtration has been reported by several authors¹³⁻¹⁵. The yields were low due to the lower concentration of IgM in serum. Molecular weight of the heavy chain of IgM was about 74 kDa under reduced condition. According to Quain Jain Fei *et al*¹⁶, the molecular weight of chicken IgM heavy and light chains were 80 and 22.5 kDa, respectively. Cadman¹⁷ reported similar values for heavy chain and light chains of chicken IgM. As per the reference¹⁴, the molecular weight of heavy chain of chicken IgM was 70 kDa. IgM light chain was not clearly visible probably due to the lower affinity to coomassie blue staining. Although reasons are not known, it has been postulated that chicken IgY light chains stain poorly with coomassie blue¹⁸.

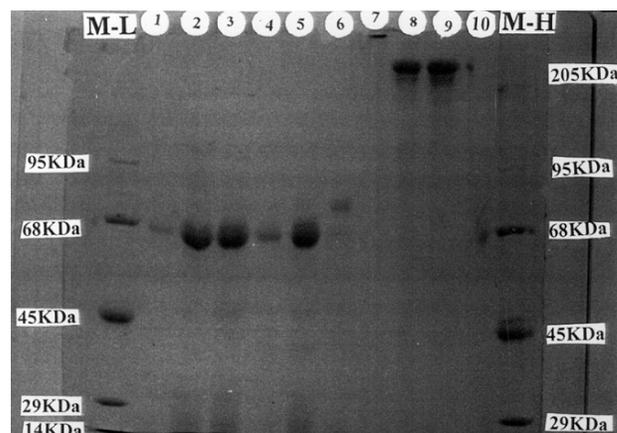


Fig. 3—SDS-Polyacrylamide gel electrophoresis of fractionated chicken immunoglobulins: 1, Standard IgY (reduced); 2-5, IgG fractions (reduced); 6, IgM fraction (reduced) 74 kDa; 7, IgM (non-reduced); 8-9, IgG (non-reduced); 10, Standard IgY (non-reduced); M-L, Molecular weight marker (low range); and M-H, Molecular weight marker (high range).

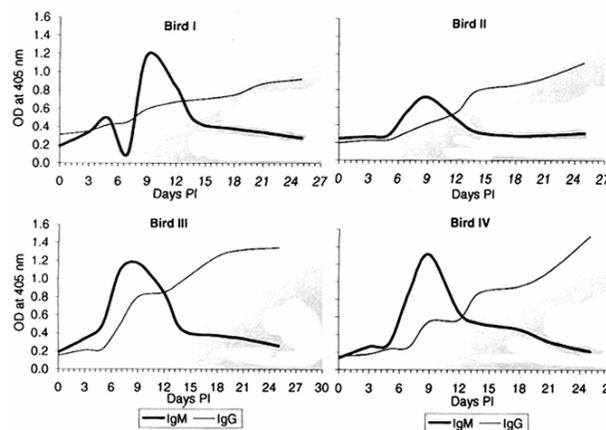


Fig. 4—Measurement of IgM response in 1-d-old chicken experimentally infected with EDS-76 virus in relation to IgG response (A representative result of four individual chickens is shown)

Isotyping of MAbs was done to determine the monoclonality and to select the stable immunoglobulin-producing clones (IgG producing clones over IgM type). In the present study, six monoclones isotyped were of class IgG1. One of the reasons might be the long immunization schedule. This finding was similar to the results reported by Erhard⁷. No cross-reactivity of the IgM MAbs with standard IgY and affinity to purified IgG fractions were noticed, indicating its purity. These results were found similar to the findings reported by Azwai¹⁹ for camel immunoglobulins.

Major application of IgM MAb produced in the study was to develop an IgM ELISA for detection of

IgM response to infectious agents and thus determine recent infections. It is considered that the presence of IgM determined the incidence of a disease, while presence of IgG indicated only its prevalence. IgM response to IBV^{2,3,5}, IBD³ and ILT³ infections has been studied. The class of antibody that is produced early in the primary response may be related to the nature of antigenic stimulus and there may be a definite sequential production of IgM and IgG²⁰. In the present study, there was an increase in IgM response in EDS virus-infected birds from 5-12 d PI, followed by predominant IgG response from 12-25 d of infection.

IgM ELISA was found to be more sensitive than the AGPT in detection of antibodies to IBD virus. In this study, only 3 samples positive by IgM ELISA were negative by AGID. In contrast, an 81% increase in sensitivity over the AGP test by the IgM ELISA has been reported for IBV⁵. It is possible that the IgM ELISA might not be more sensitive for diseases, such as IBD unlike that for IBV. It is well known that antibody responses against IBD correlate well with protection, while with respect to IBV, local antibody responses serve as a better indicator of protection than serum antibody titres. Cullen and Wyeth²¹ reported presence of precipitating antibodies against IBD with a titre of 2⁵ in 1-d-old chicks and upto 2⁹ in infected chicken. Even in the experimental/field infections, serum antibodies are detected by 3-5 d of infection by AGPT²². In the present study, only 34 field samples were taken into study, for better sensitivity results a large sample size need to be assayed.

Thus, it has been shown that the MAbs produced in this study against chicken IgM can be a valuable reagent for the detection of IgM antibodies produced in chicken serum against any poultry pathogen, simply by using the appropriate antigen-coated ELISA plates.

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