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## Production and Characterization of Monoclonal Anti-Ovalbumin Antibodies\*

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**Abstract :** In this study, the results of anti-ovalbumin (OVA) monoclonal antibody production and characterization are reported. Splenocytes from OVA-hyperimmune Balb/C mouse were fused with FO myeloma cells and resulting hybridoma cells were selected in hypoxanthine-aminopterin-thymidine containing medium. A total of 37 HAT resistant hybridomas were obtained. Upon testing the hybridoma supernatants in OVA and mouse immunoglobulin G specific enzyme-linked immunosorbent assays, we observed that two of the hybridomas secreted

OVA specific antibodies. The hybridomas produced IgG class antibodies. The hybridomas were grown and the supernatants from the cultures were also tested in a Western Blot assay. The results of ELISA were confirmed with Western Blot assays. The results indicate that the mAbs produced in this study reacted with the continuous (non-conformational) epitopes present on OVA.

**Key Words:** Ovalbumin, monoclonal antibody, hybridoma.

### Introduction

Monoclonal antibodies (mAb) have proven to be invaluable tools in various fields of biology (1). Since their first production in 1975 (2), mAbs have been used in research, medicine and industry (3). In diagnostic microbiology, almost all radioimmunoassays (RIA), enzyme linked immunosorbent assays (ELISA), and immunofluorescence assays are generated with the help of a specific mAb (4). In clinical microbiology laboratories, commercial or house-made diagnostic kits produced for almost all viral, bacterial or parasitological agents use mAb to identify antibodies or microbial antigens in clinical materials (5, 6). In clinical biochemistry and physiology laboratories, similar diagnostic kits are used to determine hormones and other clinically important molecules (5, 6). In the field of biological and biomedical research, mAbs are produced against many biologically important proteins, or to non-protein entities (7-9). These mAbs are used to investigate the structural and functional features of protein or non-protein molecules. There have been studies focusing on producing catalytic mAbs which might function as enzymes (10, 11). When such mAbs are

available in large quantities, many chemical reactions currently performed in various industrial fields will probably be catalyzed by mAbs.

The classical method for production of mAb relies on combining desirable features of a myeloma and a specific antibody-producing plasma cell (2). For this, membrane fusion of these cells is necessary. Newer methods of mAb production are based upon bacterial or eucaryotic expression of mAb-encoding genes (10, 11). Consequently, with the utilization of new methods, the immortality of myeloma cells is no longer needed.

Despite these new technological improvements, the advantages of mAb technology are not adequately used in Turkey. Almost all test kits used in clinical laboratories and mAbs needed in research laboratories are provided by outside sources. The main reason for the very limited development of mAb technology in Turkey is the fact that *in vitro* cell culturing requires expensive and laborious methodology. Currently, at Firat University, cell cultures for various cell lines are performed. Since working cell-culture conditions exist, in this study, the production and

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characterization of mAb against OVA was attempted. The reason behind the use of OVA as an antigen is the fact that OVA is a good antigen in rodents and easily available in many laboratories. This report, to our knowledge, describes one of the first studies on the production of mAb in Turkey. Similar approaches will be followed for microbiologically important agents and other molecules. The results are presented below and the implications are discussed.

### Materials and Methods

**Cells:** FO myeloma cells (kindly provided by the Institute for Molecular Biology and Genetic Engineering, TÜBİTAK, Gebze, Kocaeli) were grown in Dulbecco's Modified Eagles Medium (DMEM, Sigma Chemical Co. St. Louis, MO, USA) containing 50% nutrient mixture F-12 HAM, 10% fetal calf serum (FCS, SERVA, Feinbiochemica GmbH, Heidelberg, Germany), penicillin (100 IU/ml, Sigma), streptomycin (100 µg/ml, Sigma), and HEPES (0.1 M, Sigma).

**Immunizations:** Five 6-week-old male Balb/C mice (Institute for Molecular Biology and Genetic Engineering, TÜBİTAK, Gebze, Kocaeli) were immunized intraperitoneally (ip) with 250 mg ovalbumin (Merck, Darmstadt, Germany) in Freund's Complete Adjuvant. Two weeks later, the immunizations were repeated. In the second immunization, Freund's incomplete adjuvant was used as adjuvant. Three days prior to fusion, 250 µg OVA in 0.01M phosphate buffered saline (PBS) was given ip as the final boost.

**Fusion:** On the day of fusion, single-cell suspensions of spleenocytes from one of the hyperimmune mice were prepared and fused with FO cells as previously described (12-14). As the selective agent, hypoxanthine ( $5 \times 10^{-3}$  M)-aminopterin ( $2 \times 10^{-5}$  M)-thymidine ( $8 \times 10^{-4}$  M) was included in the media during the post-fusion culture. In addition, oxalate (0.15 mg/ml), pyruvic acid (0.05 mg/ml) and insulin (0.2 IU/ml)-containing supplement was added to the culture medium. Selection was applied until HAT resistant colonies appeared. The first colonies started to appear at around day 3 of the post-fusion period. The colonies were fed with the above-mentioned complete culture medium every 4 days. The culture supernatant of the hybridomas was tested an enzyme-linked immunosorbent assay on the 15th day (14).

Positive hybridomas were first transferred to 24-well plates and then to 25 cm<sup>2</sup> flasks. No cloning of the hybridomas was performed, because on each 96-well plate, less than 60 hybridomas were obtained.

**Enzyme-Linked Immunosorbent Assay (ELISA):** The assay procedure was similar to that described previously (15). Each well of the ELISA plates (Greiner GmbH, Frickenhausen, Germany) was first coated with 50 µl of 30 µg/ml ovalbumin dissolved in 0.05 M carbonate/bicarbonate buffer, pH 9.6, by incubating the plate at 4°C overnight. Unbound OVA was removed by washing the wells with 50 µl 0.01M phosphate buffered saline pH 7.2 (PBS) containing 0.05% Tween-20. The washing was repeated four times for each well. Afterwards, the wells were blocked with 3% horse serum (Sigma) to prevent non-specific binding. For blocking, 50 µl horse serum was added into the wells and plates were incubated at 37°C for 1hr. After incubation, the horse serum was removed from the wells and, without washing, test samples were added to the wells. The test samples contained immune/non-immune mouse sera, complete hybridoma medium and hybridoma supernatants. The serum samples were diluted 400-fold and the culture supernatants were used undiluted. All dilutions were made in 0.01M PBS, pH 7.2. The incubation and washing steps were repeated. As the reporter molecule, 1/500 diluted horseradish peroxidase conjugated goat anti-mouse immunoglobulin G (Sigma, St Louis, MO, USA) was used, and the plates were incubated and washed as described in the previous steps. The substrate solution containing o-phenylene diamine (Sigma) at 1mg/ml concentration in 0.05M sodium phosphate dibasic, 0.025 M citrate buffer (pH 6.0), which also included 0.03% H<sub>2</sub>O<sub>2</sub>, was prepared and 50 µl substrate solution was added to each well. After 15 min incubation at room temperature, the reaction was stopped by the addition of 50 µl 1N H<sub>2</sub>SO<sub>4</sub>. The color development was determined spectrophotometrically at 490 nm. The optical densities of each reaction were expressed. Along with OVA, goat anti-mouse-IgG (Sigma, St Louis, MO, U.S.A.) was also used as the solid phase antigen. Another control in the test was a negative control in which no solid phase antigen was added to the wells and the initial incubation was performed only with carbonate buffer. On each plate, positive and negative controls were included.

### Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Assay:

Ten percent SDS-PAGE was carried out as described previously (12, 16). Five  $\mu\text{l}$  samples (mouse serum and hybridoma supernatants from BG11 hybridoma and 1 mg/ml OVA in 0.01 M PBS) were boiled for two minutes in 2x sample buffer and placed in the wells. Electrophoresis was carried out in a vertical gel apparatus (OWL Sci. Inc. Cambridge, MA. U.S.A.) at 150V for 4 hr at 4°C. After the electrophoresis, one panel of the gel was stained with Coomassie Brilliant Blue as described elsewhere (16). In transferring proteins to nitrocellulose, a semi-dry blotting apparatus was used (The Wep Company, Seattle, WA. U.S.A.). Proteins were transferred under 2.5 mA/cm<sup>2</sup> for 1 hr and the nitrocellulose paper was blocked in 10 ml 5% (w/v) fat-free milk powder in 0.01M PBS for 2 hr at room temperature (12, 16). Afterwards, in one panel 1/2000 diluted OVA-immune mouse serum was used as the primary antibody. In the second panel, the primary antibody was 10 ml neat BG11 supernatant. Incubations were performed overnight at 4°C. Nitrocellulose papers were washed four times in 10 ml 0.01M PBS pH 7.2 containing 0.05% Tween-20. As the secondary antibody, 10 ml 1/1000 diluted biotinylated goat anti-mouse IgG (DAKO Corp. Carpintera, CA. U.S.A.) in PBS was added and the paper was incubated at room temperature for 30 minutes. After washing, the paper was immersed in 10 ml 1/1000 diluted streptavidin-conjugated horseradish peroxidase. In the third panel, 10 ml 1/1000 diluted horseradish-conjugated rabbit anti-mouse IgG (Sigma) was used as the primary antibody. Incubation and washing were carried out as described above. Reactions in all the panels were developed in 10 ml 0.05M Tris-HCL pH 7.6 containing 0.6mg/ml diaminobenzidine (DAB) and 0.3% H<sub>2</sub>O<sub>2</sub>.

### Results

Following the fusion, on day 3 the first colonies started to appear (Figure 1A) and at around day 10 the number of hybridoma cells increased (Figure 1B). The culture medium was replaced with fresh medium. The hybridomas were refed every three days and on day 15 the number of wells with hybridomas was determined. Accordingly, a total of 37 hybridoma colonies were observed on two 96-well plates. On one plate, a total of

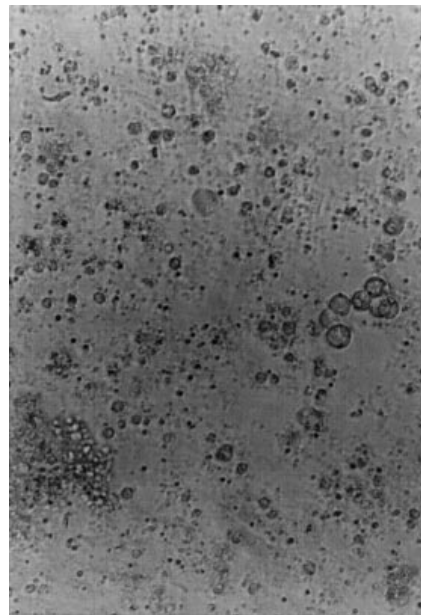


Figure 1A.

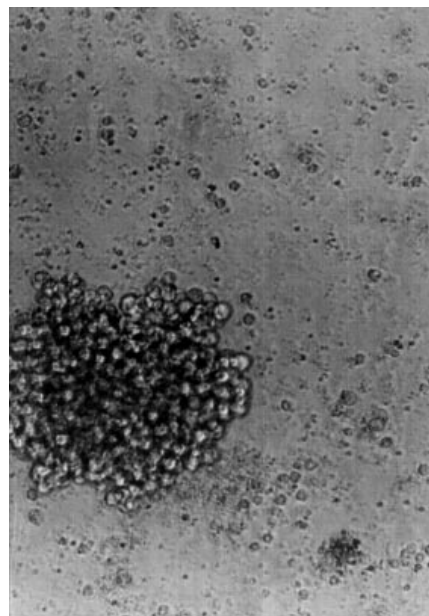


Figure 1B.

Figure 1. Light microscopic image of hybridoma colonies 3 (A) and 10 (B) days after fusion. Magnification 200x.

22 and on the other plate a total of 15 colonies were present. The hybridoma supernatants were tested in ELISA. The results are shown in Figure 2. Accordingly, two of the hybridomas were positive both in OVA and

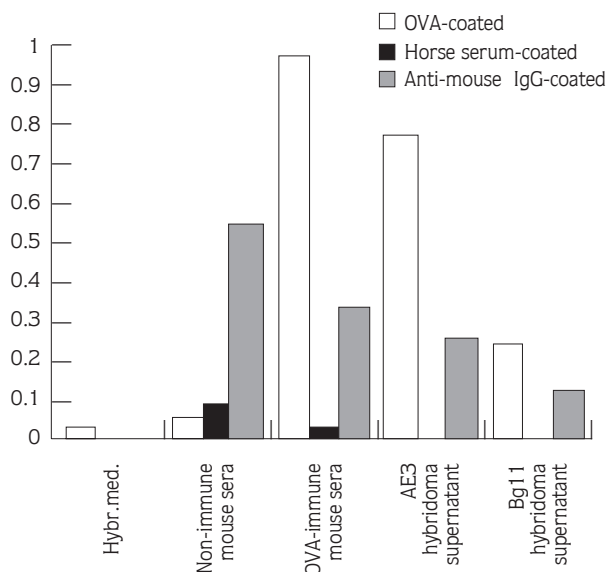


Figure 2. ELISA results of mouse anti-OVA serum and hybridoma supernatants.

anti-mouse IgG ELISA. Even though the positive hybridomas were evident even to the naked eye, the optical density of the reactions was determined

spectrophotometrically. One of the hybridomas (AE3) did not prove to be a stable cell line. After several *in vitro* passages, replication of the cells ceased and the hybridoma was lost. To confirm the ELISA results, the supernatant from the other hybridoma was tested in Western blot assays. The location of OVA on the gel was determined with Coomassie Brilliant Blue staining (Figure 3A). In the Western blot assay, 1/2000 diluted mouse anti-OVA serum was used as primary antibodies (Figure 3B). Ovalbumin specific mouse serum was tested in order to demonstrate the exact position of OVA on the paper. Thus, this assay served as a positive control to the BG11 supernatants. In a second panel, OVA specific antibodies were determined in the BG11 supernatants (Figure 3C). Since streptavidin conjugated-goat anti-mouse IgG antibodies were used as the labelling agent, naive mouse serum samples were run along with OVA (Figure 3B and C). Thus, mouse IgG present in the mouse serum served as another positive control in the Western blot assays. In Figure 3D, the presence of mouse IgG in the BG11 supernatants was determined. Here again, naive mouse serum was used as a positive control. Both the naive

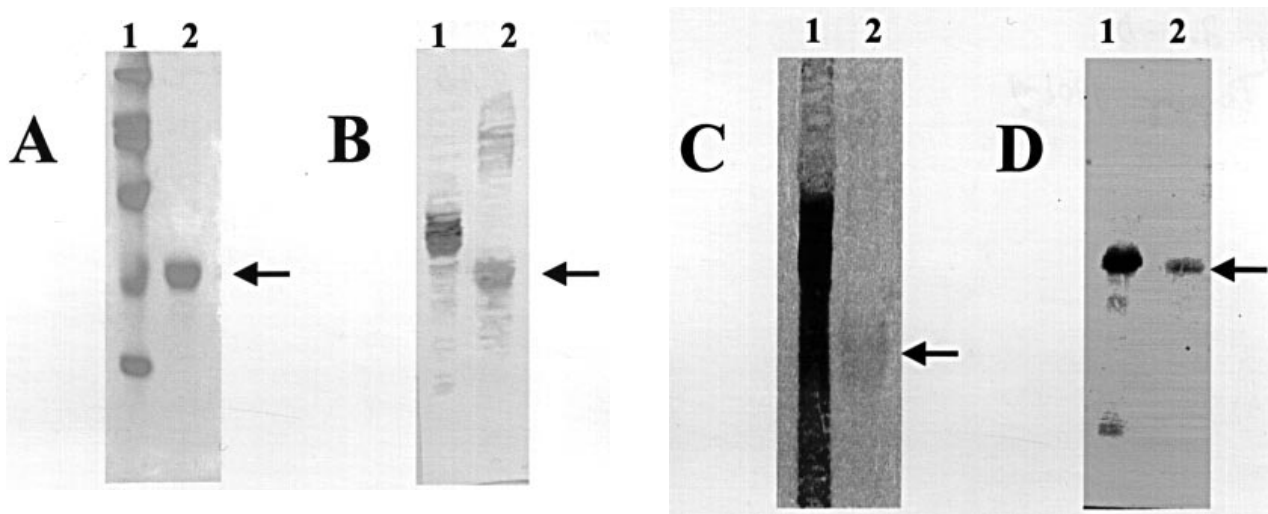


Figure 3. SDS-PAGE and Western Blot analysis of hybridoma supernatants. (A) Brilliant blue staining of the gel following SDS-PAGE showing molecular weight markers (Lane 1) and OVA (Lane 2). (B) Western Blot analysis of OVA-immune mouse serum. The naive mouse serum (Lane 1) and OVA (Lane 2) samples were electrophoresed in 10% SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods. Nitrocellulose paper was incubated with OVA-immune mouse serum. Since biotinylated goat anti-mouse IgG antibodies were used as secondary reagents, naive mouse serum containing mouse IgG was also included in the assay. Streptavidin-conjugated horseradish peroxidase was used as the labeling reagent and the reaction was developed with DAB. (C) Western Blot analysis of BG11 supernatant. The naive mouse serum (Lane 1) and OVA (Lane 2) samples were electrophoresed in 10% SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods. Nitrocellulose paper was incubated with BG11 supernatant and the reaction was developed as described in the legend for Figure 3B. (D) The presence of mouse IgG in BG11 supernatant. The naive mouse serum (Lane 1) and BG11 supernatant (Lane 2) samples were electrophoresed in 10% SDS-PAGE and transferred to nitrocellulose and horseradish peroxidase conjugated rabbit anti-mouse antibodies were used as the labeling reagent. The reaction was developed as described in Materials and Methods.

mouse serum and BG11 supernatant samples were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose and detected with horseradish-peroxidase-conjugated anti-mouse IgG. This assay also confirmed the presence of mouse IgG in the BG11 supernatant (Figure 3D).

## Discussion

Hybridoma technology has made the production of mono-specific antibodies possible in large quantities (1). A fusion event between an immortal myeloma cell and specific antibody-secreting plasma cell, when an appropriate selection is exerted, produces a hybridoma cell which has the advantages of both parents (12, 13). Thus, the hybridoma secretes specific antibodies and is immortal (13). This technology has revolutionized many disciplines in medical and biological sciences (4, 5, 8, 9).

Despite the tremendous benefits of hybridoma technology, in Turkey, researchers have been slow in applying the technology. In most studies conducted with mAb, commercial sources are used. In recent years, however, several reports on the production of mAbs have appeared in the literature (17-20). These mAbs are mostly raised against viral antigens (17-19). It is likely that similar studies from various laboratories will be published in the near future and commercial applications for these reagents will soon follow.

In this study, we produced and characterized mAb against a well-known protein, OVA. Ovalbumin induces a strong immune response in mouse and is available in pure form and in bulk. In order to establish the technology first, we chose OVA as the antigen. According to the

results, two OVA-specific antibody-secreting hybridomas were obtained. Since the mAb reacted with OVA both in ELISA and Western Blot assays, it is reasonable to assume that the epitopes recognized by the antibodies were non-conformational (21). Sodium dodecyl sulphate-PAGE is known to alter the native conformations of proteins (21). Thus, after SDS-PAGE, only linear epitopes could still be recognized. Monoclonal antibodies produced by the hybridomas were in the IgG class because in ELISA for mouse IgG, the mAbs were found to be positive.

Since secondary antibodies were specific for mouse immunoglobulins, in Western Blottings, non-immune mouse serum was included in the assay in order to provide positive controls for mouse IgG. Thus, with two panels, the presence of mouse IgG in BG11 supernatant, OVA specificity of BG11 supernatant and the exact positions of mouse IgG and OVA on each panel were demonstrated. These results were in agreement with the ELISA results, that BG11 hybridoma secreted OVA-specific mouse IgG antibodies. Further cloning for hybridomas was not attempted because only 37 colonies were present on two 96-well fusion plates. Accordingly, in cases where only one cell is plated into each well, 37% of the wells should have no growth (14). In other words, if less than 60 wells have colonies growing, there is no need to reclone the hybridomas. In this case, cells in each well originate from a single parent. Thus, they represent a monoclonal cell (14). Consequently, in this study, further cloning of hybridomas was not performed.

In future studies, mAbs against a variety of cellular or viral proteins are planned. Thus, it will be possible to use these mAbs both in clinical applications and in basic studies.

## References

1. Secher D S. Monoclonal antibodies by cell fusion. *Immunology Today*, 7:22-26, 1986.
2. Köhler G, and Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256:495-497, 1975.
3. Price P. Hybridoma technology. *Adv. Cell Culture*, 4:157-177, 1980.
4. Roitt I, Brostoff J, and Male D. Immunological Techniques. In *Immunology*, Third Edition, Mosby Year Book Europa Limited, pp 2500-2524, 1993.
5. Nowinsky RC, Tam Mr, Goldstein LC, Stong L, Kuo CC, Corey L, Stamm WE, Handsfield HH, Knapp JS, and Holmes KK. Monoclonal antibodies for diagnosis of infectious diseases in humans. *Science* 219: 637-644, 1983.
6. Joklik W K, Willet H P, Amos D B, and Wilfert C M. *Rapid Viral Diagnosis*. In *Zinsser Microbiology*, 20th edition. Appleton and Lange. pp 941-948, 1992.
7. Fowler S. Use of monoclonal antibodies for Western Blotting with enhanced chemiluminescent detection. In *Monoclonal Antibody Protocols*. Edited by W. C. Davis. Humana Press, Totowa, New Jersey. pp 115-127, 1995.
8. Shi S, Prince J B, Jones C M, Kalra K L, and Tandon A K, Use of monoclonal antibodies in immunochemistry. In *Monoclonal Antibody Protocols*. Edited by W. C. Davis. Humana Press, Totowa, New Jersey. pp 89-108, 1995.

9. Suarez C, Brown R. Use of monoclonal antibodies in immunoelectron microscopy. In *Monoclonal Antibody Protocols*. Edited by W. C. Davis. Humana Press, Totowa, New Jersey. pp 109-114, 1995.
10. Sastry L, Mubarak M, Janda KD, Benkovic SJ, and Lerner RA. Screening combinatorial antibody libraries for catalytic acyl transfer reactions. *Ciba Found. Symp.* 159: 145-151, 1991.
11. Wirching P, Ashley JA, Benkovic SJ, Janda KD, and Lerner RA. An unexpectedly efficient catalytic antibody operating by ping-pong and induced fit mechanisms. *Science* 252: 680-685, 1991.
12. Harlow E, and Lane D. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York, 1985.
13. Reading C L. Procedures for *in vitro* immunization and monoclonal antibody production. In *Hybridomas and Immortality*. Edited by B. H. Tom and J. P. Allison. Plenum Press. New York, London. pp 235-250, 1985.
14. Oi V T, and Herzenberg L A. Immunoglobulin-producing hybrid cell lines. In *Selected Methods In Cellular Immunology*. Edited by B. B. Mishell and S. M. Shilgi. W. H. Freeman and Company. New York. pp 351-372, 1980.
15. Bolat Y, Bulut H, Özdarendeli A, and Doymaz M Z. Development of Enzyme Linked Immunosorbent Assay (ELISA) for detection of anti-Infectious-Bovine Rhinotracheitis Virus (IBR) antibodies in cattle. *J. Health Sciences of Fırat Univ.* 10 (2): 283-288, 1996.
16. Sambrook J, Fritsch E F, and Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York, 1989.
17. Aybay C. Evaluation of a home-made ELISA kit for the detection of hepatitis B surface antigen (HbsAg). *Mikrobiyoloji Bült.* 33: 45-54, 1999.
18. Aybay C. Production of monoclonal IgM and IgG1 antibodies specific for hepatitis B virus surface antigen. *Gazi Medical Journal*, 10:11-15, 1999.
19. Başalp A, Yorgancı Ü, Çırakoğlu B, Bermek E. Detection of Tobacco Mosaic Virus by monoclonal antibody MAM-1F5. *Tr. J. Biology.* 21: 397-403, 1997.
20. Yücel F, Bermek E, Çırakoğlu B. Production of monoclonal antibodies specific for *Xanthoma campestris pv. phaseoli*. *Tr. J. Biology.* 21: 125-131, 1997.
21. Mahy BWJ, and Kangro HO. Western (immuno) blotting and Radio immunoprecipitation. In *Virology Methods Manual*. Academic Press. London. pp 261-265, 1996.