Production of monoclonal antibodies against recombinant nucleoprotein of avian influenza virus, serotype H9N2

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Abstract:

BACKGROUND: Avian influenza viruses (AIVs) including the subtype H9N2 cause considerable financial losses to poultry industries. Rapid and accurate diagnosis of avian influenza (AI) infection is important in control and eradication programs. **OBJECTIVES:** The aim of this study was to produce monoclonal antibodies (MAbs) specific for the nucleocapsid protein)NP (of AIV H9N2 subtype to improve diagnostic assays. **METHODS:** Recombinant NP protein was expressed in Escherichia coli and purified using amylose resin chromatography column and used as an antigen for mice immunization. Spleen cells of the immunized mice were fused with SP2/0 myeloma cells. Next, culture supernatants of primary hybridoma clones were screened by indirect ELISA. After three rounds of sub cloning, the reactivity of the MAbs with recombinant and natural antigens was assessed by Western blotting. **RESULTS:** Six MAbs showed specific binding to recombinant and natural NP from AIV H9N2 in Western blot analysis, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay. Cross-reactivity with genetically non-related including Newcastle viruse (Paramyxoviridae family) was not detected. **CONCLUSIONS:** Based on the results, the MAbs generated in this study could be used for the development of rapid diagnostic assays for recognition of AIV.

Introduction

Avian influenza is one of the main diseases with a worldwide distribution and economic losses in poultry industry. Influenza A viruses have been shown to infect a great variety of both wild and domestic poultry. Wild Waterfowl birds play an important role in the epidemiology of avian influenza viruses. In addition, AIV may cause acute and highly contagious respiratory diseases in human and other mammals (Wong and Yuen, 2006). Avian Influenza virus belongs to the Orthomyxoviridae family and the genus influenza virus. AIV are grouped into three types of A, B and C (Suarez and Schultz-Cherry, 2000). Influenza A virus genome encodes 11 viral proteins including four polymerase proteins, nucleocapsid protein (NP), hemagglutinin (HA), neuraminidase (NA), two matrix proteins (M1 and M2) and two non-structural proteins (NS1 and NS2) (Wong and Yuen, 2006). The internal antigenic determinants on the nucleoprotein

(NP) are more conserved among different subtypes, making this protein more suitable for a serological test that can detect antibodies to all AIV subtypes (Van Wyke et al., 1980). The development of rapid and accurate tests for the detection of AI is essential to its control and surveillance. MAbs are widely used as capture antibodies for detection of different important pathogens in antigen-capture ELISAs (Saijo et al., 2007; Cai et al., 2009; Qiu et al., 2009; Liu et al., 2010). Monoclonal antibodies are also used for detection of antibodies in competitive ELISAs (Martin-Folgar et al., 2010). Monoclonal antibodies against the nucleocapsid protein may be applied for the development of different diagnostic tools for AIV, for example, a competitive ELISA that can be validated for detection of AIV specific antibodies in different animal species, The use of monoclonal antibodies (mAbs) increases the specificity, accuracy and efficiency of diagnosis compared to polyclonal antibodies and provides an unlimited quantity and consistent quality of reagents (Yang et al., 2008). The main objective of this study was to generate and characterize MAbs specific for the recombinant nucleocapsid protein of AIV serotype H9N2 for use in immunodiagnostic tests for detecion of avian influenza virus antigen or antibodies.

Materials and Methods

Expression of Maltose-Binding Protein-NP Fusion Protein (MBP-NP Fusion Protein): *E.coli* BL-21 strain was transformed with plasmid pMAL-c2x-NP (for expression of MBP-NP) (Jaydari et al., 2011). Subsequently, a bacterial colony grown on LB agar medium supplemented with ampicillin was selected for expression and purification of the protein. Therefore, 5 ml of LB broth containing 50µg/ml ampicillin was inoculated with the bacteria and incubated overnight at 37 °C. The overnight cultures were used to inoculate 500 ml of fresh LB-ampicillin medium containing 2mg/ml glucose and incubated at 37 °C with shaking for about 2.5h, until the OD600 reached 0.5. Then, Isopropyl-β-D-Thiogalactoside (IPTG) (Company Cinnagen, Iran) at a final concentration of 1mM was added to the bacterial suspensions and incubation continued for an additional two h at 30 °C. Bacterial cells were harvested by centrifugation at 4000g for 10 min and suspended in 25 ml column buffer (20 mM Tris/HCl, pH7.4, 200 mM NaCl and 1mM EDTA) and stored at -20 ◦C,for future use of protein purification.

Purification of MBP-NP Fusion Protein: After expression of recombinant MBP-NP protein by pMalc2x expression vector, under the control of the lac promoter in Escherichia coli BL-21 strain, the bacterial pellet was resuspended in column buffer and sonicated to release the bacterial proteins. Purification of the expressed protein (MBP-NP) from the supernatant of the sonicated bacteria was carried out on a column of maltose-affinity chromatography based amylose resin, according to the manufacturer's instructions. In order to achieve this in the first step, the purification was performed based on MBP's affinity to amylose. Then in the second step, the MBP-NP protein was eluted from amylose resin by using 10 mM maltose solution as a competitor of amylose. Finally, the elution of recombinant protein (MBP-NP) in collected samples was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Production of Monoclonal antibodies (MAbs): For this purpose, Monoclonal antibodies were produced by immunization of five female BALB/c mice, aged 4-6 weeks (obtained from the Razi Vaccine and Serum Research Institute, Iran), with the recombinant AIV H9N2 nucleocapsid protein. The mice were immunized intraperitoneally three times at two-week intervals with 100 µg of purified recombinant AIV nucleocapsid protein emulsified in Freund's adjuvant (Sigma, St Louis, MO, USA). The first injection was with complete Freund's adjuvant (CFA). The second and third injections were performed using incomplete Freund's adjuvant (IFA), in order to stimulate a good immune response. Animals were bled two weeks after the third boost, and the final immunization was carried out three days later with 100 µg of the recombinant AIV nucleocapsid protein. Three days after the last immunization animals were bled again. The titre of the polyclonal antiserum after the final boost was assessed by indirect ELISA using recombinant AIV nucleocapsid protein as antigen (Harlow et al., 1988; Ausubel et al., 1995). The mouse with the highest titre was killed, and its spleen cells were used to fuse with Sp2/0 myeloma cells. at a ratio of 1:1 in the presence of polyethylene glycol (PEG, Sigma). Hybridoma cells were selected in RPMI media (Gibco; Gibco Invitrogen Corporation, Paisley, UK) with hypoxanthine aminopterin thymidine (HAT) (Sigma) and 5% (v/v) fetal calf serum (Gibco) and subsequently cloned by limiting dilution. Three cloning rounds were carried out to ensure that the hybridoma cell populations were positive for antibody production. For this purpose, positive clones were expanded in 24-well plates containing feeder cells and were grown overnight in a CO2 incubator. For cloning, the positive hybrids were diluted up to 8 cells/ml (in hypoxanthine thymidine; HT medium) and distributed at 100 µL/well in 96-well plates. Clones producing the highest titres of AIV nucleocapsid protein-specific antibodies, as assessed in the indirect ELISA, were selected for further use. After three rounds of cloning, positive cultures were grown to larger volumes and frozen in liquid N2, as soon as possible, in fetal bovine serum (GIBCO, Grand Island, NY) containing 10% DMSO). (Harlow et al., 1988).

Indirect ELISA: ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 2 µg/mL of recombinant MBP-NP diluted in coating buffer (0.5 M NaHO3/ Na2CO3, pH 9.6). After washing three times with PBS containing 0.05% (v/v) Tween 20 (washing buffer), plates were blocked with 50 µL washing buffer containing 5% (w/v) skim milk (Difco, Sparks, MD, USA) then incubated for 2 h at 37 °C and washed as mentioned before. Fifty microlitres of hybridoma supernatant was added to each well and incubated for 1 h at 37 °C. The plates were washed as described above followed by the addition of 50 µL of goat anti-mouse IgG peroxidase (Sigma, USA) to each well. After incubation for 1 h at 37 °C, plates were washed five times with washing buffer and 50 µL of tetramethylbenzidine (TMB) peroxidase substrate solution was added to each well. Subsequently, the plates were incubated at room temperature in the dark for 20 min, and the optical densities (OD) were determined at 450 nm in a Dynatec-MR 5000 ELISA reader.

Western blot assay: A Western blot assay was used to confirm the specificity of the clones using the recombinant AIV nucleocapsid protein and allantoic fluid of

influenza virus H9N2 inoculated eggs, and also to assess whether the MAbs recognized antigens from other viruses of the family Paramyxoviridae (Newcastle Virus). The recombinant AIV nucleocapsid protein, allantoic fluids of influenza virus H9N2 and Newcastle virus inoculated eggs were electrophoresed on 10% vertical SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were cut into strips and blocked at room temperature for 1 h with PBS containing 0.05% Tween 20 (v/v) and 5% (w/v) skim milk. Membranes were incubated with hybridoma supernatant and positive and negative controls (the same as used in ELISA) for 1.5 h at room temperature and then washed three times with 0.05% (v/v) Tween 20 in PBS. Membranes were then incubated for 1 h at room temperature with goat anti-mouse IgG peroxidase diluted 1:2000 in PBST containing 2% skim milk. After washing, the strips were developed by 4-chloro,1-naphtol (Sigma, USA) and H2O2 as the substrate. Subsequently, membranes were incubated in the dark.

Assessment of monoclonal antibodies reactivity to influenza virus by immunofluorescence assay: For immunofluorescence (IF) assay, MDCK cells grown in 96 microplates (SPL, Korea), were infected with H1N1, H3N2 and H9N2 serotypes of avian influenza virus. After 24h, the cells were fixed by adding 200 µL cold methanol to each well and incubated for 20 min at room temperature. Then, methanol was discarded and 200 µL of 0.5% Triton x-100 in PBS was added to each well to permeable the cell membranes. Following 15 min incubation at room temperature, the cells were washed three times with PBST. The supernatant of hybridoma was added and incubated for 30 min at 37 °C. Then, the cells were washed three times with PBS. Cells were incubated with fluorescent conjugated antibodies against mouse IgG (1:40 dilutions), for 30 min at 37 °C. After incubation, the cells were washed three times with PBS. Bound antibodies were visualized under a fluorescence microscope (Olympus IX71, Japan) (Harlow et al., 1988).

Results

Expression and Purification of MBP-NP Fusion Protein: After induction, the crude proteins of *E. coli* were separated by SDS-PAGE to examine whether the recombinant MBP- NP was expressed. MBP-NP fusion protein was estimated to have an approximate molecular weight of 97kDa (Fig. 1). The results showed that the recombinant AIV-NP protein was expressed successfully in *E. coli*. Subsequently, the induced protein was purified by column of amylose resin for immunization (Fig. 2).

Hybridoma Production and screening: Thirty-two hybridoma cell lines producing recombinant AIV nucleocapsid protein-specific antibodies were selected based on indirect ELISA and Western blot analysis using the recombinant AIV nucleocapsid protein as antigen. After three rounds of sub cloning, the specificity of the MAbs as confirmed by ELISA and Western blot analysis has shown that 6 of the 32 (2/G5 ،3/F6، 4/B7 ،4/D11، 5/B9 ،5/C10, 6/B3) MAbs had bound to the antigen. 6 MAbs showed a strong reaction with the recombinant AIV nucleocapsid protein (Fig.3). The MAbs were further tested for recognition of natural NP antigen in AIV using Western blotting. The same assay was used to assess the cross-reactivity of the generated MAbs with Newcastle virus. As can

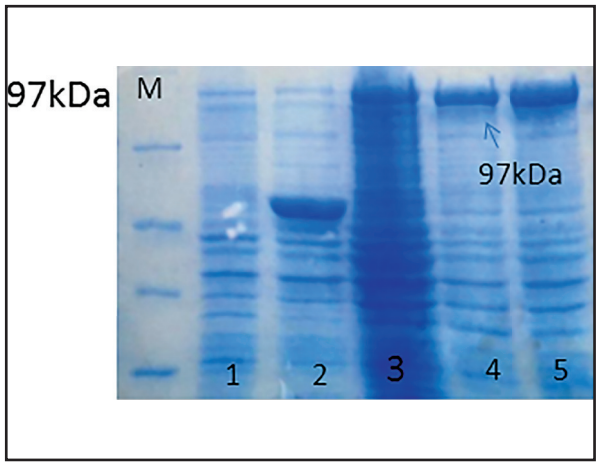

Figure 1. SDS-PAGE analysis of bacteria expressing MBP-NP fusion protein before and after induction by IPTG. Lane M: Molecular weight marker, lanes 1-5 indicate a bacterium expressing MBP before and after induction by IPTG The expression of a protein of about 97kDa, corresponding to MBP-NP, respectively.

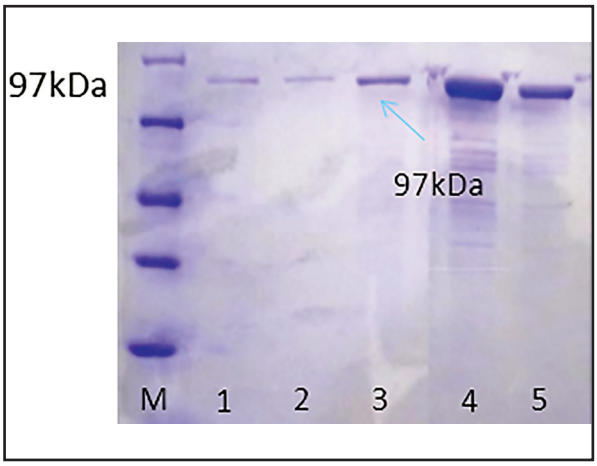

Figure 2. SDS-PAGE analysis of purified MBP-NP fusion protein by amylose-resin column. Lane (1-5) shows purified nucleoprotein of influenza virus (~97 KDa).The molecular weight marker is shown in Lane M.

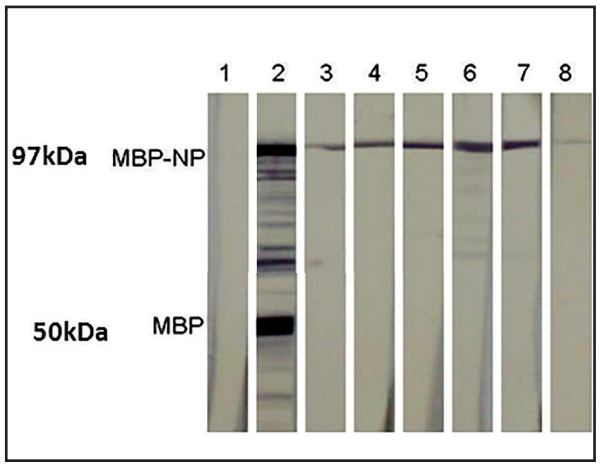

Figure 3. Western blotting screening of different hybridoma supernatants with recombinant AIV nucleocapsid protein. lane 1: anti-recombinant AIV nucleocapsid protein serum; lane 2: serum from non immunized mouse; lanes 3-8: different hybridoma supernatants.

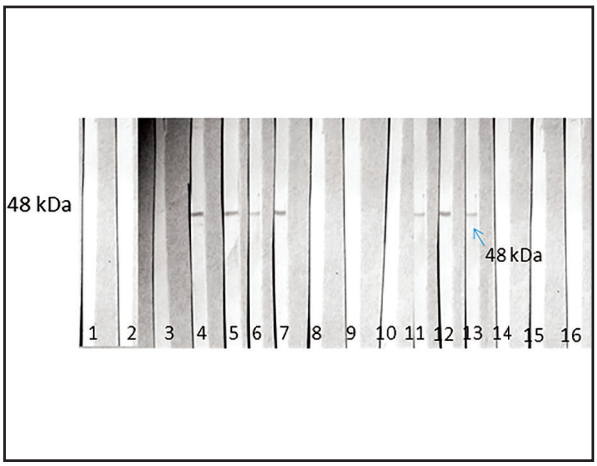

Figure 4. Western blot analysis of MAb specificity. Inactivated AIV and Newcastle virus were probed with MAb. Lanes 1-3: indicate supernatant of an unrelated hybridoma. Lanes 4-7,11-13: represent supernatant of subclones containing anti-NP mAb to AIV. Lanes 8-10, 14-16: Cross reactivity of supernatants of subclones producing anti-NP mAbs to Newcastle virus. The arrow indicates positive reaction (48 kDa band).

be seen in Fig.4, the monoclonal antibodies specifically recognized the AIV produced an expected band of approximately 48kDa. However, no reaction was observed, neither on other viral extracts nor on the negative control extract. All six MAbs proved specific for AIV nucleocapsid protein. Also, Immunofluorescence assay was performed on MDCK cells infected with avian influenza virus serotypes H1N1, H3N2 and H9N2 to assess whether the mAbs recognize the native-form of NP protein of AIV. MAbs strongly reacted with MDCK infected cells (Fig.5A) while uninfected cells showed no reaction to any mAbs (Fig.5B).

Discussion

Avian influenza (AI) has been identified as an etiological infectious agent with serious

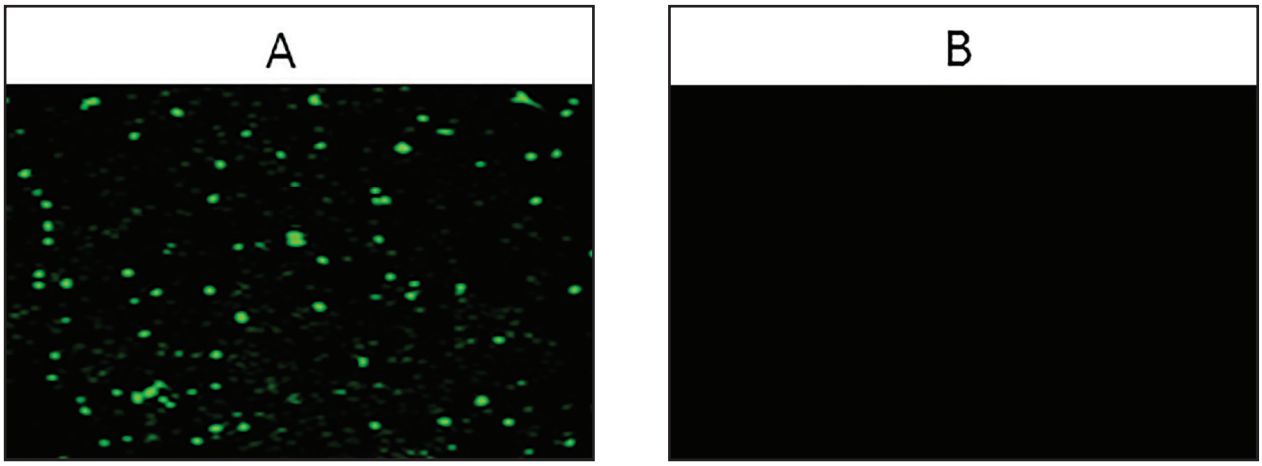

Figure 5. Reactivity of supernatants of subclones producing anti-NP mAbs to natural NP antigen by indirect immunofluorescence assay. A: MDCK cells infected with Serotypes: H1N1, H3N2 and H9N2. B: MDCK cells, No special fluorescence was found on normal cells.

economic loss. Finding effective methods to control and prevent the virus infection is important. The development of new rapid and accurate diagnostic tools is needed to detect antibodies against AI virus (AIV) proteins (Watson et al., 2009). Avian influenza NP is an internal antigenic determinant and preserved among different subtypes and strains of influenza A virus and has been applied successfully in indirect ELISA test for the detection of AIV-specific antibodies (Kang et al., 2006; Wu et al., 2007; Yang et al., 2008). In the present study, for the first time production of a large amount of an immunologically active recombinant NP protein of AIV H9N2 subtype in a prokaryotic expression system, under the control of the strong promoter (lac) of pMalc2x vector enabled us to produce anti-NP MAbs by cell fusion assay. The MAbs revealed a specific ability to recognize the recombinant nucleocapsid protein both based on an indirect ELISA and recognition of antigens in a Western blot assay. Reactivity of the MAbs with Native NP protein was established by immunofluorescence and Western blotting. Six of 32 chosen MAbs based on ELISA results gave a strong reaction in Western blot. To assess whether the produced MAbs were specific for AIV only, they were tested with other RNA viruses from the family Paramyxoviridae (Newcastle Virus). No cross-reactivity was found using western blot for analyzing the six selected MAbs. Monoclonal antibodies against AIV were previously described by Yang et al. (2008, 2010), and Bhat et al. (2013). Yang et al. (2008) developed MAbs against recombinant nucleocapsid protein AIV H5N1 subtype. Two MAbs were tested in a slot blot for their reactivity against 15 subtypes of influenza virus; F28-73 reacted with all tested 15 subtypes, while F26-9 failed to react with H13N6 and H15N8. Yang et al (2010) produced MAbs after immunization of BALB/c mice with the whole-inactivated AIV H7N1. Six of seven MAbs reacted with viruses of the H7 subtype, but not with any of the other s hemagglutinin (HA) subtypes tested. Bhat et al. (2013) produced MAbs after immunization of BALB/c mice with recombinant nucleocapsid protein AIV H5N1. Two of 11 MAbs reacted with recombinant nucleocapsid protein. Based on the results, the MAbs generated in this study could be used in a competitive ELISA to detect specific AIV

antibodies in the sera from different species. This could provide rapid diagnostic assays for detection of AIV and reduce its distribution between animals and humans.

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چکیده

زمینه مطالعه: ویروس‌های آنفلوانزای پرندگان شامل تحت تیپ H۹N۲سبب ضررهای اقتصادی قابل ملاحظه‌ای به صنعت طیور می شوند. تشخیص سریع و دقیق عفونت آنفلوانزای پرندگان در برنامه‌های ریشه کنی و کنترل این بیماری بسیار مهم می‌باشد. **هدف:** هدف ازاین مطالعه تواید آنتی بادی‌های منوکلونال اختصاصی نوکلئوپروتئین ویروس آنفلوانزای پرندگان تحت تیپ H۹N۲ برای بهبود و بیشرفت روش‌های تشخیصی بود. **روش کار:** پروتئین نوترکیب NP در باکتری *E.coli* بیان گردید و با استفاده از ستون کروماتوگرافی رزین آمیلوز خالص سازی و به عنوان یک آنتی ژن برای ایمن سازی به موش تزریق شد. فیوژن سلول‌های طحال با سلول‌های میلوما انجام شد. در مرحله بعد مایع رویی کشت سلولی کلون‌های هیبریدوما اولیه به وسیله الایزای غیر مستقیم غربالگری شدند. بعد از سه بار کلونینگ واکنش آنتی بادی‌های منوکلونال با آنتی ژن‌های طبیعی و نوترکیب به وسیله وسترن بلات تأیید شد. **نتایج:** شش آنتی بادی منوکلونال اتصال اختصاصی به نوکلئوپروتئین نوترکیب و طبیعی در ویروس آنفلوانزای برندگان تحت تیپ H۹N۲ در وسترن بلات' الیزا و ایمونوفلورسانس را نشان دادند. واکنش متقاطع با ویروس‌های غیر مرتبط از لحاظ ژنتیکی از جمله ویروس نیوکاسل (خانواده پارامیکسوویریده) تشخیص داده نشد. **نتیجه‌گیری نهایی:** براساس نتایج آنتی بادی‌های منوکلونال تولید شده در این مطالعه می‌توانند برای طراحی آزمایشات تشخیصی سریع برای شناسایی ویروس آنفلوانزای پرندگان استفاده شوند.

واژه‌های کلیدی: آنتی بادی، پرندگان، آنفلوانزا، منوکلونال، نوکلئوپروتئین

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