Iran Red Crescent Med J. 2019 October; 21(10):e95906. doi: 10.5812/ircmj.95906.
Published online 2019 November 3. **Research Article**

Monoclonal Antibody Preparation Against Nucleoprotein of Avian Influenza Virus Subtype H9N2

Maryam Hashemi [1], Rasool Madani [2,*], Mahmoudreza Aghamaali [3], Tara Emami [2] and Fariba Golchinfar [2]

[1]Department of Biology, University Campus 2, University of Guilan, Rasht, Iran
[2]Department of Proteomics and Biochemistry, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran
[3]Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran

[*]*Corresponding author*: Department of Proteomics and Biochemistry, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran. Email: madanirasool@gmail.com

Received 2019 July 02; **Revised** 2019 October 03; **Accepted** 2019 October 08.

Abstract

Background: In recent years, various poultry diseases have posed risks to this industry. Respiratory and infectious diseases are the most common diseases. According to previous findings, influenza disease is recognized as a significant life-threatening disease in the industry of poultry worldwide. Influenza virus type A, which belongs to the family *Orthomyxoviridae*, is responsible for a serious infectious disease. H9N2 AVI subtype circulates in the poultry worldwide, causing significant economic losses and infections in humans, also domestic and wild animals.
Objectives: The purpose of the present experimental research was monoclonal antibodies (MAbs) production in contradiction of the NP of avian influenza virus (AIV) H9N2 subtype to detect AIV antigens and antibodies in the Department of Proteomics and Biochemistry, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran. in 2017.
Methods: The conserved protein of NP from H9N2A/Chicken/Iran/259/2014 virus, with 60 kDa molecular weight, was isolated using the electroelution method. The purified protein was applied for Australian BALB/c mice immunization. After evaluating immunization by ELISA assay, the spleen of immunized mice was isolated and hybridized with SP2/0 myeloma cell. Next, the hybridized cell was cultured, and clone soups were collected after 15 days to examine antibodies via ELISA assay. The produced antibodies using Western blotting and antibody isotype kits were characterized.
Results: Thirty antibody-producing clones were examined for reactivity against Nucleoprotein (NP), the antigen, at 1 μg/mL concentration. According to the ELISA assay of antibody titers, two (3/F10 and 2/D7) out of 30 antibodies were bound to the antigen with titer 0.863 and 1.641, respectively. Two hybrid clones, 2D7 and 3F10, which produced anti-NP antibodies, were isolated and cultured. Characterizing of the produced antibodies using Western blotting was performed using H9N2 virus; finally, two clone soups (3/F10 and 2/D7) reacted with the NP virus protein. According to the isotyping of antibodies produced by 3F10 and 2D7 clones, 3F10 clone produced IgG1 with a κ chain, and IgG1 concentration was 1.997, based on ELISA assay. Also, 2D7 clone produced IgG2a with a κ chain, and IgG2a concentration was obtained 1.951.
Conclusions: According to the findings of our study, the produced antibody might be used in the diagnosis of influenza.

Keywords: Avian Influenza Virus, H9N2 Subtype, Inbred BALB C, Influenza A Virus, Influenza in Birds, Mice, Monoclonal Antibody, Nucleoprotein (NP), Poultry Diseases

1. Background

In recent years, various poultry diseases have posed risks to this industry. Respiratory and infectious diseases are the most common diseases, which are caused by microbial factors, such as viruses, bacteria, and fungi. According to previous findings, influenza disease is recognized as a significant life-threatening disease in the industry of poultry worldwide (1).

Avian influenza virus (AIV) H9N2 strain was isolated from turkey in the United States in 1966, firstly (2). Though viruses of H9 are categorized as low-pathogenicity avian influenza viruses (LPAI), a fatality rate above 50% has been reported (3) and making it difficult for poultry veterinarians to diagnose (4).

H9N2 AVI subtype circulates in the poultry worldwide, causing significant economic losses and infections in humans, also domestic and wild animals (3, 5, 6). H9N2 AIV

Copyright © 2019, Author(s). This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (http://creativecommons.org/licenses/by-nc/4.0/) which permits copy and redistribute the material just in noncommercial usages, provided the original work is properly cited

can transmit from birds to humans and other mammals such as pigs (7-10). H9N2 human infections have been incessantly reported, but there were no H9N2 vaccines available in humans (11). Generally, AIV from the family *Orthomyxoviridae* is polymorphic, with a single-stranded, eight-part RNA genome (12). Type A is an epidemic factor and known as the most virulent and contagious in poultry influenza (13).

At present, the most appropriate strategy for influenza management is early detection and control (14). An important part of influenza control programs is the accurate and rapid detection of viruses in different animal species. Respiratory illnesses have similarities to the symptoms; therefore, diagnosis is important issue in the management of this pandemic (15). Currently, serological diagnostic techniques, such as agar gel propagation (AGP), enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition (HI), are usually applied as important tools for antibodies detection towards specific viral antigens (16). Generally, effective detection and monitor are necessary for the influenza infection management (17).

The results of some studies have shown that internal proteins, such as NPs, are one of the significant influenza A virus determinant; therefore, NP is a specific type of antigen, useful for influenza viruses A, B, and C detection. It is known that NP is a highly conserved antigen among a variety of influenza viruses (18). Accordingly, isolation of conserved NPs from H9N2 viruses and preparation of monoclonal antibodies against NPs can be helpful in identifying the virus in different species, based on serological methods (19-22) and competitive ELISA assay (23).

Use of monoclonal antibodies against conserved proteins, such as NP, can be introduced as an appropriate strategy for immediate detection of influenza virus in a variety of animal species; this method can increase the diagnosis efficiency and accuracy, in comparison with polyclonal antibodies (24). NPs, which are suitable for serological tests given their conservation among different influenza A virus strains (25, 26), can detect antibodies to all AIV subtypes.

2. Objectives

In the current research, monoclonal antibodies (mAbs) were manufactured and characterized against the purified H9N2 NP for use in immunodiagnostic tests.

3. Methods

3.1. Viruses

AIV strain A/flash chicken/Iran/772/1998 (H9N2) was acquired from the Department of Influenza Reference Laboratory, Razi Vaccine and Serum Research Institute (Karaj, Iran) and stored at -70°C in 2017.

3.2. Isolation of NP Antigen via Gel Electrophoresis

In this experimental study, that performed in the Department of Proteomics and Biochemistry, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran, Standard bovine serum albumin (BSA) protein (Sigma, St. Louis, MO, USA) was used, based on the Lowry method to determine the amount of proteins in the viral solution (27). For analyzing proteins with biological activity, a non-denaturing system is required. For this purpose, a non-denaturing system, according to the Davis method, was used to purify proteins (28, 29). NP was separated via polyacrylamide gel electrophoresis (PAGE). A Model 422 Electro-Eluter (Bio-Rad) was also used based on the manufacturer's guidelines to recover NP.

The concentration of purified protein was defined by the Lowry method (27) at an optical density (OD) of 750 nm, and protein quantity was calculated by using BSA (Sigma, USA) as standard. The purified NP protein (60 kDa) was finally analyzed based on Tricine-SDS-PAGE method (30). It should be noted that Tricine-SDS-PAGE gels are usually applied to segregate proteins below 100 kDa.

3.3. Immunization with NPs

Two female BALB/c mice (4 - 6 weeks), acquired from Razi Vaccine and Serum Research Institute (Iran), subcutaneously were immunized with 40 μg of purified NP, mixed in a complete Freund's adjuvant (Sigma, USA). Also, using the same protein in incomplete Freund's adjuvant (Sigma, USA), three booster vaccinations were performed within three-week intervals. Blood sampling was performed one week after the third boost (31). The polyclonal antiserum titer was measured after the final immunization via indirect ELISA, using AIV NP as antigen (32).

3.4. Generation and Selection of Hybridoma Cells

The SP2/0-Ag14 myeloma cell line expansion was accomplished in RPMI-1640 medium, supplemented with 20% fetal bovine serum (FBS) one week before fusion. On the day of cell fusion, the myeloma cells total number in the spleen of the mouse was 1×10^8 cells in two flasks. Final immunization was carried out with the same antigen in saline at a dose of 28 μg via intravenous injection three to four days before fusion. One day before fusion, a non-immunized BALB/c mouse spleen cells with Sp2/0 myeloma cells were sowed in 96-well plates of hybridoma cells to support the growth of hybridoma cells.

On the day of fusion, myeloma cell line SP2/0-Ag14 was maintained in the log phase and examined by an inverted microscope to assure sufficient growth of cells for fusion. Next, spleen cells of mouse were applied to fuse with Sp2/0 myeloma cells at a 4:1ratio in the 50% polyethylene glycol (PEG; Sigma) presence. The cell suspension (100 μL) was gently aspirated to every well of 96-well flat-bottom plate and at 37°C incubation in a 5% CO_2 incubator.

Afterward incubation during three days, the wells were examined under an inverted microscope. Next, 100 μL of hypoxanthine aminopterin thymidine (HAT; Sigma) and 20% (v/v) FBS in RPMI medium (Gibco Invitrogen Co., Paisley, UK) were added to each well and placed in a 5% CO_2 incubator at 37°C. On days 2, 4, 6, 8, and 10, the cells were fed with HAT, and on day 12, the feeding protocol was repeated using hypoxanthine-thymidine (HT) medium. From day 16 onwards, the wells were not fed with HAT or HT. The hybridomas were prepared to screen, as the majority of wells containing the grown cells demonstrated the confluence of 10% - 25%.

3.5. Screening of Secreted Antibodies by ELISA Assay

Hybridoma supernatants containing the grown cells in the wells were screened using an NP antigen via indirect ELISA assay. NP antigens were coated onto a Maxisorp microtiter plate (Nunc, Roskilde, Denmark) at an optimal concentration of 1 μg/mL and overnight incubation at 4°C in a coating buffer (0.1 M carbonate/bicarbonate; pH: 9.6). Next, the antigen-coated plate was blocked with 200 μL/well of blocking buffer (5% non-fat dry milk in PBST) for 90 minutes. Then, 100 μL of hybridoma supernatant was added to every well with 75 minutes incubation. In addition, 100 μL of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (1:1000; Sigma, USA) was added to each well, and the plate was incubated for 75 minutes. Finally, the plate was incubated with 100 μL of BM Blue POD substrate (Roche Diagnostics GmbH, USA) for 20 minutes in darkness at room temperature.

By adding 0.1 M sulfuric acid the reaction was terminated, and OD was read at 450 nm by a microplate spectrophotometer (BioTek Instruments Inc., USA). Every step of incubation was conducted at 37°C after that with three times plate washing using a washing buffer (0.01 M PBS; pH: 7.5), comprising 0.05% (v/v) Tween-20 (PBST). Hybridoma supernatants, which tested positive on NP-based ELISA assay, were transferred to a 24-well plate and grown in a CO_2 incubator. Then, positive clones were grown to larger volumes and immediately frozen in liquid N_2 in FBS (Gibco, USA), containing 10% DMSO.

3.6. Characterization of mAbs Against NP

3.6.1. ELISA Assay

ELISA assay was used to screen clone antibodies by coating NP with the concentration obtained using the checkerboard method. The clone soup was evaluated to determine the antibody titer.

3.6.2. Western Blotting

Western blotting was applied to approve the reactivity of different mAbs using AIV H9N2 subtype. The H9N2 virus protein solution was subjected to 10% SDS-PAGE under reduced conditions. Next, protein electrophoresis was performed and protein bands onto a nitrocellulose membrane, by a Trans-Blot semi-dry transfer cell (BioRad, USA) were transferred. After blocking the blotted membrane in 3% BSA solution, it was incubated for 90 minutes at room temperature. The membrane was washed with PBST three times for 10 minutes. Then, with the hybridoma supernatant, the membrane was incubated overnight at 4°C. With PBST for three minutes, the membrane was washed five times and then incubated again for one hour in a rabbit anti-mouse IgG secondary antibody (HRP-conjugated; 1:1000 in 5% BSA). After washing the membrane, it was immersed in a precipitating substrate solution, containing chloronaphthol for 30 minutes until the protein band appeared.

3.7. Determination of Antibody Isotypes

By a mouse mAb isotyping kit (Amersham Bioscience, UK) and ELISA assay isotyping were performed. ELISA assay was conducted based on the formerly explained method with an NP protein concentration of 1 μg/mL. Then, anti-mouse IgG2a antibodies, and anti-mouse IgG, anti-mouse IgG1were added to the conjugated peroxidase phase, and the rest of the procedure was carried out similar to the indirect ELISA method.

4. Results

4.1. NP Protein Purification

Based on the Lowry method, the protein amount in the solution of purified protein was 0.296 mg/mL. The analysis results of Tricine-SDS-PAGE demonstrated only one protein band with a 60 kDa molecular weight (Figure 1).

4.2. Hybridoma Cell Production and Checkerboard Method

To obtain the optimal antigen concentration for evaluation of monoclonal antibodies, checkerboard method was applied. For this purpose, serial concentrations of NP protein were coated, and serum samples of immunized

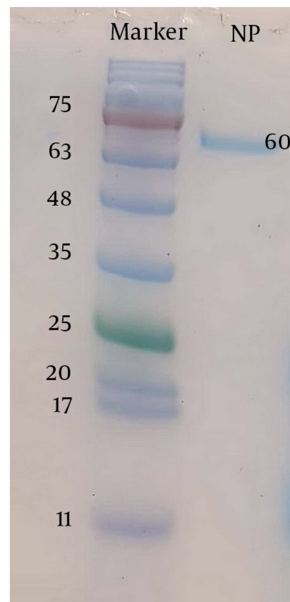

Figure 1. Analysis of Tricine-SDS-PAGE of purified NP protein

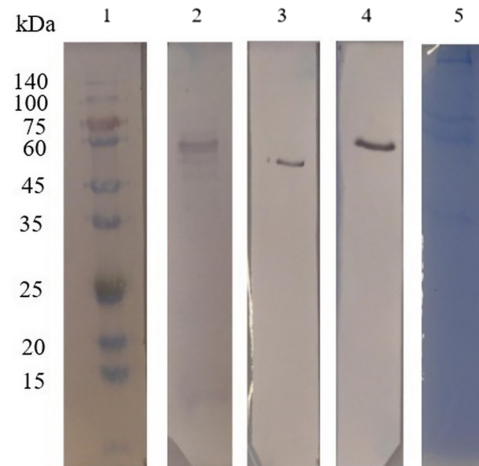

Figure 2. Western blotting screening of different hybridoma supernatants via the analysis of SDS-PAGE of AIV H9N2 subtype. Lane 1: molecular weight marker; lane 2: serum of an immunized mouse with H9N2; lane 3: 2D7 hybridoma supernatant; lane 4: 3F10 hybridoma supernatant; and lane 5: SDS-PAGE results of AIV H9N2 subtype.

mice were evaluated at different dilutions against the positive control serum. All experiments were done triplicated. The optimal antigen concentration was found to be 1 μg/mL (Table 1).

4.3. Screening of Clones for Antibody Production by ELISA Assay

Thirty antibody-producing clones were examined for reactivity against NP (antigen) at 1 μg/mL concentration, which was determined based on the checkerboard method using indirect ELISA assay. All experiments were done triplicated. According to the ELISA assay of antibody titers, two (3/F10 and 2/D7) out of 30 antibodies were bound to the antigen (Table 2).

4.4. Western Blotting of Hybridized Clones and Polyclonal Sera

Western blotting was performed using H9N2 virus, which was subjected to 10% SDS-PAGE under reduced conditions. As can be seen, two clone soups reacted with the NP virus protein. Also, Western blotting of polyclonal serum was performed with several immune response protein bands (Figure 2).

4.5. Isotyping of Antibodies Produced by 3F10 and 2D7 Clones

Isotyping of antibodies produced by 3F10 and 2D7 clones was examined quantitatively using isotyping and ELISA assays. According to the qualitative method, 3F10 clone produced IgG1 with a κ chain, and IgG1 concentration was 1.997, based on ELISA assay. Also, 2D7 clone produced IgG2a with a κ chain, and an IgG2a concentration was obtained 1.951 (Figures 3 and 4 and Table 3).

5. Discussion

Avian influenza is a significant viral disease worldwide. H9N2 viruses have relatively fewer pathogenesis types, whereas over the past decade, there have been many reports of damage or loss in these strains (3, 14, 33). NP is the most important influenza virus internal protein, which can help detect influenza viruses A, B, and C. Previously research have shown that NP can be a proper target for the antiviral drugs development against influenza virus different types by stimulating immune responses (34, 35).

AIV NP is conserved between different subtypes and strains of the influenza virus (36). In the present study, NP was purified using the electroelution method on the Native PAGE gel. Native gel was used to preserve the biological activity and native structure of the protein. Considering the low amount of viruses, methods such as chromatography were not applicable for purifying the protein. Generally, the technique used in the present study is applicable not only for soluble proteins, but also for membrane proteins. The separation of different proteins by this method has been also reported in previous studies (37).

The protein that purified was evaluated using Tricine-SDS PAGE, and a 60-kDa band was observed. Therefore, the immune cells of spleen were hybridized with myeloma SP2/0 cells. Since the mice were immunized with NP, all

Table 1. Results of Checkerboard Method for Determining the Optimal Antigen Concentration of NP Protein

Dilution of sera ↓	Serum of mice ↓	0.25 μg/mL of NP antigen	0.5 μg/mL of NP antigen	1 μg/mL of NP antigen	2 μg/mL of NP antigen
1:20	Mouse 1	0.532	0.799	0.991	0.968
	Mouse 2	0.630	0.854	0.932	1
	Control+	1.624	1.813	2.117	2.161
1:40	Mouse 1	0.498	0.680	0.736	0.718
	Mouse 2	0.574	0.636	0.591	0.593
	Control+	1.01	1.139	1.183	1.217
1:80	Mouse 1	0.358	0.407	0.470	0.366
	Mouse 2	0.378	0.495	0.588	0.337
	Control+	0.987	0.856	0.9	0.988

Figure 3. Isotyping of antibodies produced by 3F10 and 2D7 clones based on the qualitative method

Figure 4. Isotyping of antibodies produced by 3F10 and 2D7 clones based on the qualitative method

clones producing antibodies were directed against NP. In addition, ELISA assay was used to screen the antibodies of clones by coating the NP protein. Clones secreting antibodies were cultured and transferred to new flasks. Frequent screening of 30 clones based on ELISA assay indicated two clones with high antibody titers. Also, clone F10 from plate 3 and clone D7 from plate 2 were cultured in new flasks, and their antibodies were evaluated. In addition, the anti-

Table 2. Screening of Clones for Antibody Production by ELISA Assay

	Clone Screening/NP-Coated, 1 μg/mL
1F4	0.167
1F8	0.208
1E9	0.175
1E7	0.198
1D8	0.172
1B4	0.182
1C9	0.171
1G11	0.173
2H8	0.166
2G3	0.161
2F11	0.364
2E9	0.189
2D7	1.641
2D5	0.215
2C9	0.403
1F7	0.188
1D2	0.197
1B8	0.176
1G3	0.185
3F2	0.178
3F3	0.176
3F10	0.863
3C11	0.182
3D10	0.191
3D8	0.192
3G8	0.165
3H7	0.176
3C5	0.188
3F8	0.176
3C6	0.17
Positive control	2.442
Negative control	0.2

Table 3. Isotyping of Antibodies Produced by 3F10 and 2D7 Clones Based on ELISA Assay

Isotyping IgG	Total IgG	IgG1	IgG2a
3F10	1.64	1.997	0.125
2D7	2.649	0.126	1.951
Positive	1.033		
Negative	0.096		

bodies were characterized based on Western blotting, and then, antibody isotypes were identified.

HI test is the most common method for evaluating antibody titers against influenza virus. This test evaluates the increase in antibody titers against the virus by controlling blood clots. In this test, antibodies act against HA of viruses and prevent the binding of virus by the red blood cells. However, this process is relatively insensitive for antibody detection (38).

Although NP is a reasonably well-preserved AIV protein with antigenic variations, it occurs much more slowly than HA and NA proteins (39). Analysis of phylogenetic of virus strains was derived from various hosts discovered that the NP gene might vary up to a maximum amino acid difference of 11% (40). Therefore, it is essential to apply NP antigens derived from circulating AIV strains for the mAbs development in order to increase the sensitivity of mAb-based diagnostic assays.

In the study, it is the first time that native NPs antibodies purified from mice were investigated. Recombinant NPs don't have post-translational modifications and often have some additional amino acids which interrupt the production of native antibodies, but it is a time-consuming process. Generally, mAbs against NP protein is a key part in the diagnosis of AIV infections (41). In the current research, mAbs were developed against type A-specific NPs, derived from the H9N2 subtype, for their probable usage in detection tests of antigen or antibody against all type-A influenza viruses in poultry. The findings showed that mAbs against NP could be useful in the development of the immunochromatographic tests for detection of antigen or competitive ELISA assays for diagnosis of AI antibody in the poultry.

Footnotes

Authors' Contribution: Maryam Hashemi, Rasool Madani and Mahmoudreza Aghamaali developed the original idea and the protocol, abstracted and analyzed data. Maryam Hashemi, Tara Emami and Fariba Golchinfar contributed to the development of the protocol, abstracted data, and prepared the manuscript.

Conflict of Interests: The authors of the present review declare no conflict of interest.

Ethical Approval: 95002-9251-18-18-12.

Funding/Support: This study was supported by Department of Proteomics and Biochemistry, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran.

References

1. da Silva MC, Schaefer R, Gava D, Souza CK, da Silva Vaz I Jr, Bastos AP, et al. Production and application of anti-nucleoprotein IgY antibodies for influenza A virus detection in swine. *J Immunol Methods*. 2018;**461**:100–5. doi: 10.1016/j.jim.2018.06.023. [PubMed: 30158073].
2. Homme PJ, Easterday BC. Avian influenza virus infections. I. Characteristics of influenza A-turkey-Wisconsin-1966 virus. *Avian Dis*. 1970;**14**(1):66–74. doi: 10.2307/1588557. [PubMed: 4314007].
3. Ji K, Jiang WM, Liu S, Chen JM, Chen J, Hou GY, et al. Characterization of the hemagglutinin gene of subtype H9 avian influenza viruses isolated in 2007-2009 in China. *J Virol Methods*. 2010;**163**(2):186–9. doi: 10.1016/j.jviromet.2009.09.013. [PubMed: 19781574].
4. Nili H, Asasi K. Avian influenza (H9N2) outbreak in Iran. *Avian Dis*. 2003;**47**(3 Suppl):828–31. doi: 10.1637/0005-2086-47.s3.828. [PubMed: 14575072].
5. Li H, Liu X, Chen F, Zuo K, Wu C, Yan Y, et al. Avian influenza virus subtype h9n2 affects intestinal microbiota, barrier structure injury, and inflammatory intestinal disease in the chicken ileum. *Viruses*. 2018;**10**(5). doi: 10.3390/v10050270. [PubMed: 29783653]. [PubMed Central: PMC5977263].
6. Pan Q, Liu A, Zhang F, Ling Y, Ou C, Hou N, et al. Co-infection of broilers with Ornithobacterium rhinotracheale and H9N2 avian influenza virus. *BMC Vet Res*. 2012;**8**:104. doi: 10.1186/1746-6148-8-104. [PubMed: 22748160]. [PubMed Central: PMC3424113].
7. Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, Xu KM, et al. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin Microbiol*. 2005;**43**(11):5760–7. doi: 10.1128/JCM.43.11.5760-5767.2005. [PubMed: 16272514]. [PubMed Central: PMC1287799].
8. Lin YP, Shaw M, Gregory V, Cameron K, Lim W, Klimov A, et al. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. *Proc Natl Acad Sci U S A*. 2000;**97**(17):9654–8. doi: 10.1073/pnas.160270697. [PubMed: 10920197]. [PubMed Central: PMC16920].
9. Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, et al. Human infection with influenza H9N2. *Lancet*. 1999;**354**(9182):916–7. doi: 10.1016/s0140-6736(99)03311-5. [PubMed: 10489954].
10. Xu C, Fan W, Wei R, Zhao H. Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. *Microbes Infect*. 2004;**6**(10):919–25. doi: 10.1016/j.micinf.2004.04.015. [PubMed: 15310468].
11. Lim YH, Phan LV, Mo IP, Koo BS, Choi YK, Lee SC, et al. Production and characterization of monoclonal antibodies against an avian influenza virus (H9N2). *Korean J Vet Service*. 2017;**40**(3):187–92.
12. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Mol Biol Rev*. 1992;**56**(1):152–79.
13. Yoo SJ, Kwon T, Lyoo YS. Challenges of influenza A viruses in humans and animals and current animal vaccines as an effective control measure. *Clin Exp Vaccine Res*. 2018;**7**(1):1–15. doi: 10.7774/cevr.2018.7.1.1. [PubMed: 29399575]. [PubMed Central: PMC5795040].
14. Hay AJ, Skehel JJ, McCauley J. Characterization of influenza virus RNA complete transcripts. *Virology*. 1982;**116**(2):517–22. doi: 10.1016/0042-6822(82)90144-1. [PubMed: 7064346].
15. Moghadami M, Kazeroni PA, Honarvar B, Ebrahimi M, Bakhtiari H, Akbarpour M. Influenza A (H1N1) virus pandemic in Fars province: A report from southern Iran, July-December 2009. *Iran Red Crescent Med J*. 2010;**12**(3):231–8.
16. Yang J, Dai X, Chen H, Teng Q, Li X, Rong G, et al. Development of blocking ELISA for detection of antibodies against H9N2 avian influenza viruses. *J Virol Methods*. 2016;**229**:40–7. doi: 10.1016/j.jviromet.2015.12.011. [PubMed: 26724274].
17. Linke S, Neubauer K, Dorner MB, Dorner BG, Pauli G, Schweiger B. Generation and characterisation of monoclonal antibodies against influenza virus A, subtype H5N1. *J Virol Methods*. 2011;**175**(1):85–94. doi: 10.1016/j.jviromet.2011.04.025. [PubMed: 21549148].
18. Yang M, Berhane Y, Salo T, Li M, Hole K, Clavijo A. Development and application of monoclonal antibodies against avian influenza virus nucleoprotein. *J Virol Methods*. 2008;**147**(2):265–74. doi: 10.1016/j.jviromet.2007.09.016. [PubMed: 18006085].
19. Saijo M, Georges-Courbot MC, Marianneau P, Romanowski V, Fukushi S, Mizutani T, et al. Development of recombinant nucleoprotein-based diagnostic systems for Lassa fever. *Clin Vaccine Immunol*. 2007;**14**(9):1182–9. doi: 10.1128/CVI.00101-07. [PubMed: 17634509]. [PubMed Central: PMC2043324].
20. Cai JP, Wang YD, Tse H, Xiang H, Yuen KY, Che XY. Detection of asymptomatic antigenemia in pigs infected by porcine reproductive and respiratory syndrome virus (PRRSV) by a novel capture immunoassay with monoclonal antibodies against the nucleocapsid protein of PRRSV. *Clin Vaccine Immunol*. 2009;**16**(12):1822–8. doi: 10.1128/CVI.00244-09. [PubMed: 19828768]. [PubMed Central: PMC2786387].
21. Qiu LW, Di B, Wen K, Wang XS, Liang WH, Wang YD, et al. Development of an antigen capture immunoassay based on monoclonal antibodies specific for dengue virus serotype 2 nonstructural protein 1 for early and rapid identification of dengue virus serotype 2 infections. *Clin Vaccine Immunol*. 2009;**16**(1):88–95. doi: 10.1128/CVI.00212-08. [PubMed: 19020106]. [PubMed Central: PMC2620663].
22. Liu M, Chen X, Wang Y, Zhang Y, Li Y, Wang Y, et al. Characterization of monoclonal antibodies against Muscovy duck reovirus sigmaB protein. *Virol J*. 2010;**7**:133. doi: 10.1186/1743-422X-7-133. [PubMed: 20569474]. [PubMed Central: PMC2907335].
23. Martin-Folgar R, Lorenzo G, Boshra H, Iglesias J, Mateos F, Borrego B, et al. Development and characterization of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein generated by DNA immunization. *MAbs*. 2010;**2**(3):275–84. doi: 10.4161/mabs.2.3.11676. [PubMed: 20400862]. [PubMed Central: PMC2881254].
24. Nguyen LT, Nakaishi K, Motojima K, Ohkawara A, Minato E, Maruyama J, et al. Rapid and broad detection of H5 hemagglutinin by an immunochromatographic kit using novel monoclonal antibody against highly pathogenic avian influenza virus belonging to the genetic clade 2.3.4.4. *PLoS One*. 2017;**12**(8). e0182228. doi: 10.1371/journal.pone.0182228. [PubMed: 28787440]. [PubMed Central: PMC5546692].
25. Schild GC, Oxford JS, Newman RW. Evidence for antigenic variation in influenza A nucleoprotein. *Virology*. 1979;**93**(2):569–73. doi: 10.1016/0042-6822(79)90259-9. [PubMed: 88124].
26. van Wyke KL, Yewdell JW, Reck LJ, Murphy BR. Antigenic characterization of influenza A virus matrix protein with monoclonal antibodies. *J Virol*. 1984;**49**(1):248–52. [PubMed: 6197538]. [PubMed Central: PMC255448].
27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;**193**(1):265–75. [PubMed: 14907713].
28. Davis BJ. Disc electrophoresis. Ii. method and application to human serum proteins. *Ann N Y Acad Sci*. 1964;**121**:404–27. doi: 10.1111/j.1749-6632.1964.tb14213.x. [PubMed: 14240539].
29. Walker JM. Nondenaturing polyacrylamide gel electrophoresis of proteins. *The protein protocols handbook*. Springer; 2009. p. 171–6. doi: 10.1007/978-1-59745-198-7_20.
30. Schagger H. Tricine-SDS-PAGE. *Nat Protoc*. 2006;**1**(1):16–22. doi: 10.1038/nprot.2006.4. [PubMed: 17406207].
31. Harlow E, Lane D. *Antibodies: A laboratory manual+ cold spring harbor*. New York: Cold Spring Harbor Laboratory Press; 1988.
32. Ausubel F, Kingston R, Moore D, Seidman J, Smith J, Struhl K. *Current protocols in molecular biology*. 2nd ed. Massachusetts: John Wiley& Sons. Inc; 1995.
33. Mariana MB, Ketherson RS, Maria FSM, Filipe SF, Maria LFT, Romeu MS, et al. Development and application of an enzyme-linked immunosorbent assay (ELISA) using a soluble recombinant nucleoprotein for the

detection of antibodies to avian influenza virus. *Afr J Microbiol Res*. 2017;**11**(18):697–704. doi: 10.5897/ajmr2016.8407.

34. Hagiwara K, Kondoh Y, Ueda A, Yamada K, Goto H, Watanabe T, et al. Discovery of novel antiviral agents directed against the influenza A virus nucleoprotein using photo-cross-linked chemical arrays. *Biochem Biophys Res Commun*. 2010;**394**(3):721–7. doi: 10.1016/j.bbrc.2010.03.058. [PubMed: 20230782].
35. Kao RY, Yang D, Lau LS, Tsui WH, Hu L, Dai J, et al. Identification of influenza A nucleoprotein as an antiviral target. *Nat Biotechnol*. 2010;**28**(6):600–5. doi: 10.1038/nbt.1638. [PubMed: 20512121].
36. Kang HM, Jeong OM, Kim MC, Kwon JS, Paek MR, Choi JG, et al. Surveillance of avian influenza virus in wild bird fecal samples from South Korea, 2003-2008. *J Wildl Dis*. 2010;**46**(3):878–88. doi: 10.7589/0090-3558-46.3.878. [PubMed: 20688693].
37. Dunn MJ. Electroelution of proteins from polyacrylamide gels. *Methods Mol Biol*. 2004;**244**:339–43. doi: 10.1385/1-59259-655-x:339. [PubMed: 14970570].
38. Stephenson I, Wood JM, Nicholson KG, Charlett A, Zambon MC. Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/Singapore/97 vaccine. *Virus Res*. 2004;**103**(1-2):91–5. doi: 10.1016/j.virusres.2004.02.019. [PubMed: 15163495].
39. Starick E, Werner O, Schirrmeier H, Kollner B, Riebe R, Mundt E. Establishment of a competitive ELISA (cELISA) system for the detection of influenza A virus nucleoprotein antibodies and its application to field sera from different species. *J Vet Med B Infect Dis Vet Public Health*. 2006;**53**(8):370–5. doi: 10.1111/j.1439-0450.2006.01007.x. [PubMed: 17010040].
40. Shu LL, Bean WJ, Webster RG. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. *J Virol*. 1993;**67**(5):2723–9. [PubMed: 8474171]. [PubMed Central: PMC237595].
41. Yang M, Clavijo A, Suarez-Banmann R, Avalo R. Production and characterization of two serotype independent monoclonal antibodies against foot-and-mouth disease virus. *Vet Immunol Immunopathol*. 2007;**115**(1-2):126–34. doi: 10.1016/j.vetimm.2006.10.002. [PubMed: 17109972].