ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)

Vol 11, Issue 3, March 2024

Generation of Murine Hybridomas Producing Anti-Newcastle Disease Virus-Specific Monoclonal Antibodies

[1] Didhiti Agarwal

[1] Amity Institute of Biotechnology, Amity University, Maharashtra, Mumbai - Pune Expressway, Bhatan, Post-Somathne, Panvel, India

Corresponding Author Email: [1] didhitiagarwal2000@gmail.com

***Abstract*— Antibodies generated by a unique clone of B cells are called monoclonal antibodies, and mAbs from the exact B cell clone bind to the identical epitope(s). In diagnostics and therapeutics, mAbs serve as an excellent tool. NDV is a highly contagious pathogen causing nervous, respiratory, and enteric diseases amongst birds worldwide. Since poultry farming is done chiefly in rural areas, developing cheap, easy, and effective diagnostic kits, such as rapid antigen detection kits, can be helpful in the quick and easy detection of NDV infection.**

Therefore, this study is predicted on generating the NDV-specific hybridoma that can be used to produce mAbs suitable for developing rapid antigen detection kits. For generating the NDV- specific B cells, three mice were immunized subcutaneously with NDV after getting the required permission from Institutional Animal Ethics Committee (IAEC, SSBS, SIU). After the secondary and booster immunization, hemagglutinin inhibition assay (HIA) was performed with NDV specific antibodies and serum of experimental animals. The mouse expressing the highest antibody titer, primed with NDV immunization four days before sacrificing it. Splenocytes were prepared from sacrificed mice spleen and fused with SP2/0 myeloma cells using PEG and electrical pulse. The fused hybridoma cells were grown on hypoxanthine- aminopterin - thymidine (HAT) media. Cultured cells HIA supernatant was utilized for detection of NDV-specific antibodies. Two out of four cultures were positive for the particular antibody and it was observed that, IgG level was elevated in the serum whereas, IgM + IgA were significantly elevated in the supernatant. Hybridoma producing anti-NDV specific antibodies have been generated. Successful expansion of NDV specific hybridoma clones will lead to the development of monoclonal antibodies that can be used for making the lateral flow assay kit suitable for rapid virus detection.

Keywords: Hybridoma, Monoclonal Antibody, Newcastle Diseases Virus (NDV), ELISA, Rapid detection kit.

I. INTRODUCTION

Disease is derived from an old french "desaise," meaning distress, sickness, and discomfort. Today, despite increased technology and research with the production of various vaccines across the globe, major disease outbreaks still prevail, like SARS-CoV-2 in humans and Newcastle Disease Virus (NDV) in animals. Rapid detection kits are needed for immediate detection to control and stop the spread of such diseases. Lakhs of farmers face significant losses due to the disease's spread and inadequate survival equipment.[1]

Despite widespread vaccination, (NDV), a paramyxovirus pervasive to most places around the globe, causes a deadly disease in chicken. In chickens, pathogenic NDV strains leads to Newcastle disease (ND), a devastating systemic illness causing 100% mortality.

NDV is an enclosed virus with -vely polarized RNA as its genomic material. The outer spike glycoprotein of Influenza A virus (F) is composed of Hemagglutinin-neuraminidase (HN), six structural proteins and various fusion proteins (F proteins), employed for attachment and adsorption in host cell. These proteins which promote attachment to and subsequent entry into the host cell, are encoded by the nucleotide genome. The F protein is type-I integral membrane protein, major contributors of NDV virulence. Hence, the World Organization for Animal Health (OIE) has put it on its list of illnesses requiring rapid notification upon recognition and early detection of NDV.[2,3] Technological and industrial advances enable the production of novel derivatives, biosimilars, and approved antibodies. [4] More than 700 antibody based drugs are in clinical trials. Antibodies are the most important part of the humoral immune system, which guards against invading pathogens such as viruses and bacteria. IgG possesses the highest therapeutic potential of all isotypes, accounting for 70– 80 percent of total antibodies.

Combining an antibody-producing B cell with an immortal myeloma cell generates hybridoma cells. The hybridoma production approach produces functional, highly specific, and highaffinity mAbs. Various therapeutic monoclonal antibodies (mAbs) were generated with hybridoma technology and, being employed for the diagnosis, prevention, and treatment of various disorders. [5]

Later following quite a while of exploration and extraordinary review novel jobs for a considerable length of time harmfulness determinants are as yet being intuited. Generation of highly specific, functional monoclonal antibodies with great affinity towards immunogenic antigens through mice hybridoma technology involves various steps including optimization, splenocyte extraction, fusion of

ISSN (Online) 2456 -1304
International Journal of Science, Engineering and Management (IJSEM)
Vol 11, Issue 3, March 2024

splenocytes with immortalized myeloma cells. [6] The optimization of antibody secretion as a part of host innate immune response the Ag adjuvant needs to be administered in host for weeks. After confirmation of immune response, splenocytes are extracted with high titer binding Abs against the specific immunized antigen.[7] Fusion of splenocytes and immortalized myeloma cells is mediated by fusogenic agents. Myeloma cells are vulnerable to HAT media because they lack the HGPRT gene, which is required for de novo or salvage nucleotide synthesis. However, unfused B cells die due to their limited lifespan [8] only hybrid (B cellmyeloma) cells carry the functional HGPRT gene from B cells; hence, only hybrid cells survive.

Even after the development of better technological advancements in monoclonal antibody synthesis, hybridoma is still the most preferred option due to its aboriginal ability to conserve original antibody information and safeguard immune cell innate activities. Recent breakthroughs in antibody engineering have permitted the development of monoclonal antibodies without species level constraints. This has resulted in generation of conserved, nonimmunogenic [9] human protein targeting monoclonal antibodies.

Recombinant technology has overcome the difficulties of unstable hybridomas by cloning the transfection vectors in mammalian systems to generate target specific antibodies. The hybridoma approach is the oldest, most successful, and most essential method for isolating mAbs.[10] The hybridoma-derived antibodies preserve the native coupling of variable and constant region genes, which promotes research into the direct and indirect activities of a monoclonal antibody. In contrast, the NDV is notable due to the fact that it multiplies more rapidly in malignant cells as compared to healthy human cells and is capable of destroying these host cells.[11] NDV can be administered to cancer cells directly or as a cancer vaccine.

Vaccinations against cancer stimulate the immune system to seek out and destroy cancer cells. Despite inconclusive results from clinical trials (research studies involving humans) of NDV as a cancer therapy, it is currently in use., the medicine is now in development. Detection and further research on the subject make it more significant.[12]

II. MATERIALS AND METHODS

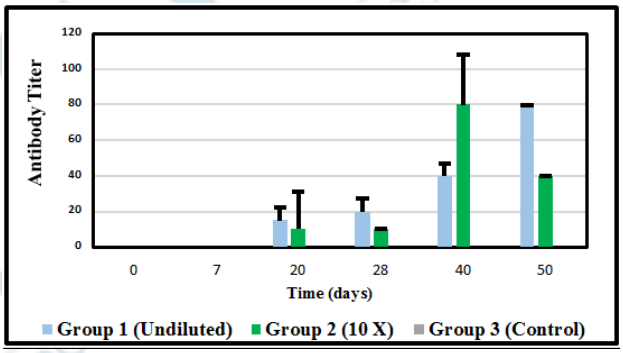
Isolation of NDV: The isolated strains of Newcastle Disease Virus were provided from the animal facility house, SIU, Pune.

Hemagglutination assay of the isolates was carried out and were inactivated using 2% formaldehyde respectively. 24 hrs after incubation at 25°C on the shaker. The stock was purified and stored for further studies.[13] **Confirmatory assays for detection of inactivated NDV:** The inactivation of the virus was carried on the basis of the titer value and significant levels of contaminants, such as immunogenic chicken host cell proteins by Allantoic fluid assay and 12- well seeding.[14,15]

Allantoic fluid assay: 1mL of inactivated virus was injected in the 10–11-day old Spf embryo and incubated at 37°C for 4 days. The egg was opened using forceps and the allantoic fluid was collected and was centrifuged at 2000 rpm for 5mins. The supernatant was tested for titer value by HA.

12-well seeding: Vero cell line was revived and passaged in a T25 flask. After 90% confluency, the cells were seeded in a 12 well plate in a ratio of 1:3 and 50 µL of the inactivated virus was added to each well. The plate was incubated for 4 days at 37°C and later viewed under the microscope for morphological changes.

Immunization: 8-10 weeks old BALB/C mice were immunized intraperitoneally with 100 µL of fluid containing 1 x 10^5 FFU of the virus. After immunization on the 7th day, the serum of the mice was collected and screened for the amount of antibodies formed. During the course of immunization, various antibodies were formed at different levels in different groups. As the number of doses increased; the formation of antibodies also increased although the groups showed different levels at different time periods.



Graph 1: Antibody formation during the course. The serum from the mice was titrated using HAI assay.

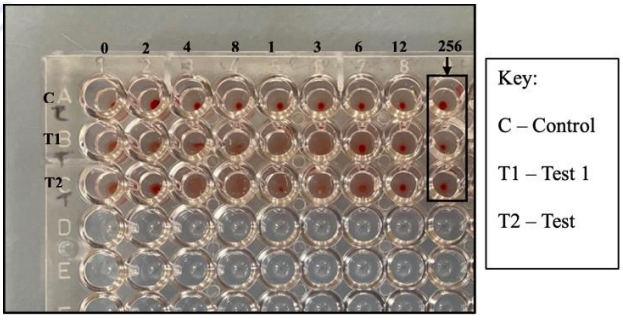


Figure 1: Microplate HA of NDV

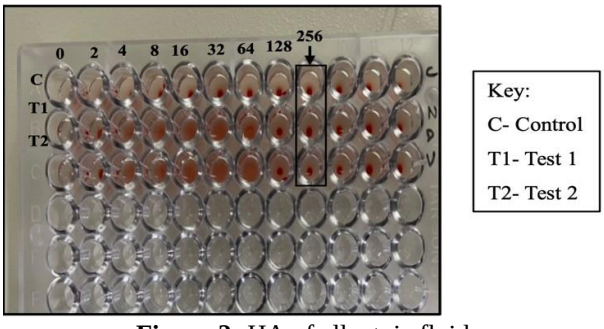


Figure 2: HA of allantoic fluid

ISSN (Online) 2456 -1304
International Journal of Science, Engineering and Management (IJSEM)
Vol 11, Issue 3, March 2024

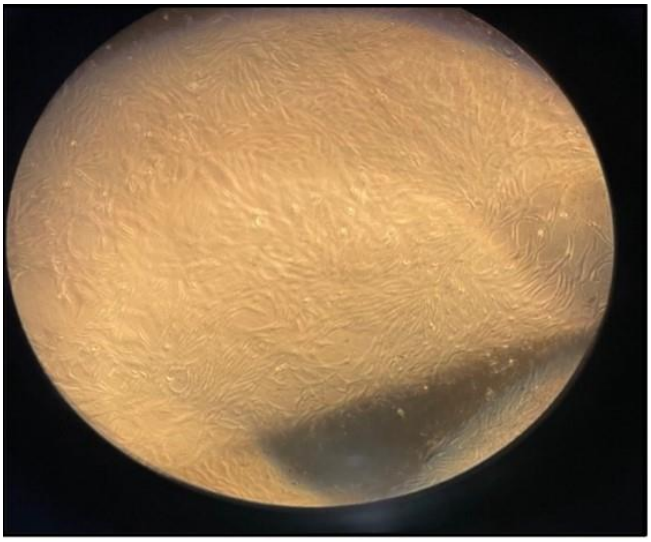


Figure 3: Vero cell line infected with inactivated NDV

Analysis of serum: To examine the generation of specific antibodies, per mouse 200 µL of serum was collected and transferred at 4°C for 45 mins. Later, centrifuge at 3000 rpm for 5 mins at 4°C and pipette out the clear fluid in a fresh tube.

Hemagglutination Inhibition Assay : The HA assay for the virus suspension was carried out in order to know the titer of the virus suspension. Once the virus titer was known, dilution of the virus was carried out to get a titer of 4 HAU. A 96 well round U- bottom plate by Thermo ScientificTM was used and each column was labelled respectively, with columns 10,11 and 12 in each plate as the control. To each of the wells, starting from column 2 up to column 11 in the 96 well plate 25 µL of 1X PBS was added, while in the 12th column, 50 µL of 1X PBS was added along with 50 µL of serum. Serially 1:10 dilution was carried out till the 9th well, and then 25 µL was discarded. 25 µL of the antigen was added to all wells from column 1 to column 9. Tap the plate for a homogenous mix and incubate it at room temperature for 10 mins. [14] Immediately, 50 µL of 1% RBC was added to all wells in the plate. The plate was covered and gently tapped and it was incubated at room temperature for 25 minutes. The titer value was recorded.

Preparation of Feeder cells: SP2/0 cell line was revived with complete media (RPMI-1640 supplemented with 10 % FBS, L-glutamine, and 50µM mercaptoethanol) in a T25 flask. After 90% confluency, the cells were passaged for generating hybridoma cells.

Hybridoma Production: An injection of a particular antigen into a mouse results in the extraction of antigen-specific plasma cells (antibody-producing cells) from the mouse's spleen, which are then fused with a malignant immune cell known as a myeloma cell to form a hybridoma.[16]

Cell suspension: The immunized mouse was sacrificed by isopropanol inhalation, the spleen was excised, and it was collected in plain media. The excised spleen was washed with distilled water to remove all the unwanted RBC and with the help of forceps, the spleen was squeezed through the 50mm cell strainer. All the contents were transferred to a falcon tube, and 10 mL of complete media was added. Cells were harvested and centrifuged at 200 x g, 10 min, and 4 °C, pellet was washed with plain media. In the falcon tube, Hisep in 1:3 ratio was added along with PBS via the walls. The solution was centrifuged at 720 x g for 20 mins at room temperature with brakes off in swinging bucket rotor. The white layer was observed in the interphase. This interphase was collected very gently, and it was washed with 5 mL of plain media. The suspension was centrifuged at 1500 rpm for 10 minutes at RT.

After discarding the supernatant, the pellet was resuspended in 1 mL of complete media.

Carry of the cell count process for both; the myeloma cells as well as a the splenocytes.\

Fusion of cells: From the cell suspension, 200 µL was taken and mixed with 200 µL of PEG8000. The entire 400 µL mixture was transferred into an electroporation cuvette with a diameter of 2 mm, and the pulse was set at 600 V and 25 m/s and incubate at RT for 3 mins. Repeat the fusion 4x until the entire 1 mL batch has been spent. The cells were transferred to the feeder layer T25 flasks (the spleen cells formed the feeder layer). The suspension was supplemented with 65 µL of HAT Medium. The flask was incubated at 37°C in a 5% CO2 incubator with 6% - 7% humidity. The ultimate concentrations should be 100 M hypoxanthine, 5.8 M azaserine, and 16 M thymidine in the flask within the well. 7days later, clonal growth was examined microscopically (magnification: 20X objective lens, 20X eyepiece) to determine whether it was monoclonal or polyclonal. The complete media from the flasks were changed 7 days later. The cells were incubated again for 7 days in HAT medium. [17,18]

Limiting dilution of hybridomas: Dilution of the positive hybridomas was carried out in such a manner that each mL has 10 cells only (10 cells/mL). A 96 well cell culture plate by Thermo ScientificTM was used. The plate was first seeded with feeder cells and cell count of the suspension was carried out. Based on the count obtained, the cell number was adjusted to such that only 10 cells/ mL were there. It was incubated at 37°C in a 5% CO2 incubator with 6% - 7% humidity for 7 days. After 7 days replicating samples were pooled for further evaluation. The HI assay, for all the replicating samples was carried out and the samples were studied further.

The positive clones were screened by ELISA and later they were purified using columns.

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)

Vol 11, Issue 3, March 2024

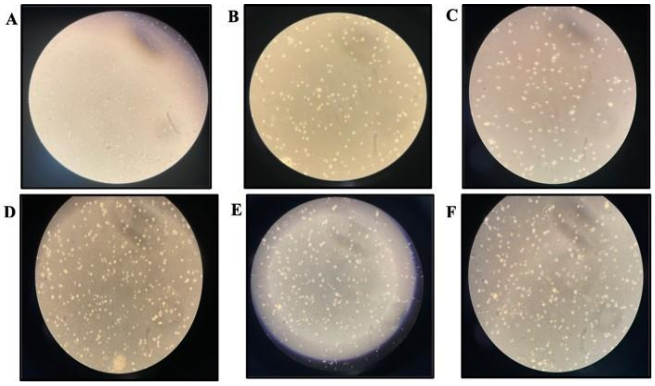
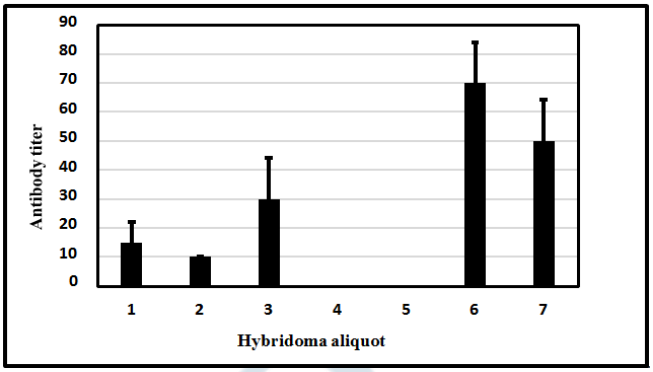


Figure 4: A-C: Control flask 1, 2 & 3 show no monoclonal hybridomas formed on day 1 after cell fusion. D-F: Monoclonal hybridomas were analysed microscopically under 20X, 7 days after cell fusion. The images depict monoclonal hybridoma clones.

ELISA: Coating of the Corning's Costar™

ELISA plate with 100 µL of capture antibody in 1X Coating Buffer. [19] The plate was sealed with parafilm and incubated at 4°C overnight. Adding 1 mL of Assay buffer A to 9 mL of 1X PBS yielded the 2X Blocking Buffer. It was adequately aspirated from the wells and washed twice with 200 µL/well of washing Buffer. The plate was blotted with absorbent paper to eliminate any buffer residue. Blocking the wells with 125 µL of Blocking Buffer. The plate was incubated for two hours at room temperature. During incubation, the Standard Antibody was produced by reconstituting 200 ng/mL of mouse IgG in distilled water. The detection Antibody was made by diluting 1:250 in 1X Assay Buffer. Wash the plate as in the previous step above and repeat for a total of two washes. 50 µL of 1X Assay buffer was added to standard wells and 50 µL of the reconstituted standard was added in duplicates into well G8 and H8. The contents of the well were mixed by repeated aspiration and 50 µL was transferred to the next wells and discarded from the last well. 100 µL of the sample was added to all the wells as per the plan (prediluting them at least 10,000-fold 1:100 in 1X Assay Buffer A). The dilution of other biological samples needs to be determined empirically. 50 µL/well of diluted detection antibody was added to all wells in the plate. The plate was sealed with parafilm and was incubated at RT for 2 hours on a microplate shaker set at 150 rpm. Wash the plate as done above and repeat for a total of four washes. In each well, 100 µL of Substrate Solution was added, and the plate was incubated at RT for 15 minutes. Add 100 µL of Stop Solution to each well. The was read at 450 nm.

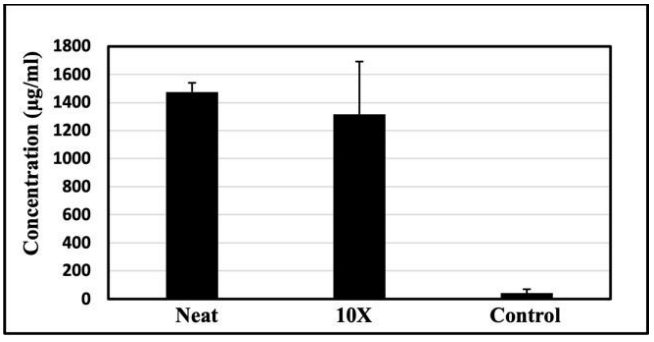


Graph 2: Antibody formation in the hybridoma aliquots. The supernatant from the mice were titrated using the HAI assay.

Purification of MAbs: The monoclonal hybridomas with the appropriate ELISA results were tested. The culture supernatant was centrifuged at 2000 rpm at 4°C for 15 min and filter it through a 0.45-µm filter. Protein A based spin column by HiGenoMB was used for IgG purification. The column's lower cap was removed and placed in the 2 ml collecting tube and centrifuge for 1min at 500 X g. Equilibrate the spin column with 0.4 ml of Binding Buffer and centrifuge for 1min at 500 X g, discard the flow through. For the application of the Sample, the spin column's exit was sealed with a cap and

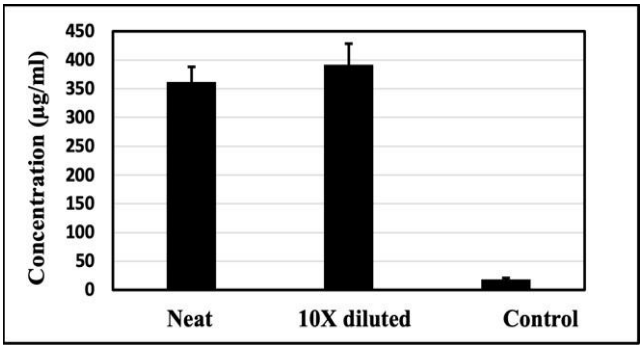
0.5 ml of the sample (containing the immunoglobulin to be purified) was introduced through the spin column's top. The sample and resin were in contact for 30 mins before the bottom cap was removed. It was centrifuged for 1 min at 500 X g, and the flow through was collected. The spin column was transferred to a fresh collection tube, and 0.4 ml of binding buffer was injected through the top to remove any proteins not retained by the column. The column was centrifuged for 1 min at 500 X g, and the flow through was collected in new tubes. After adding 0.4 ml of Elution Buffer, the lid was sealed. The column was well mixed for 10 mins before the bottom cap was removed. It was centrifuged for 1 min at 500 X g, and the eluate was collected. This process was done twice, yielding a total of three distinct eluates. By adding 40 µL of Neutralization Buffer to each 0.4 ml of eluted fraction, each fraction was neutralised. The protein concentration was measured at 280 nm.

Note: Rinse the spin column with Binding Buffer until the OD 280 nm of the washes returns to the initial level. [20,21]

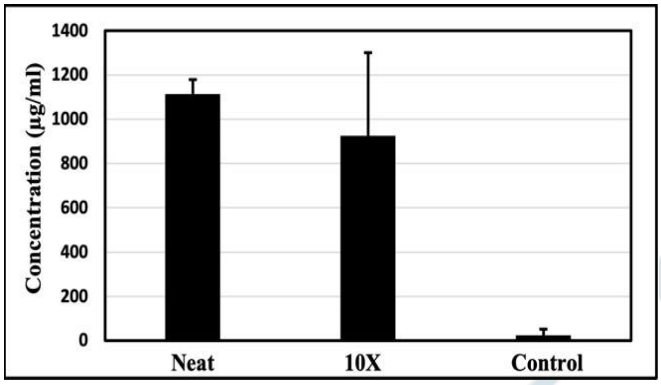


Graph 3: Total NDV specific Antibodies

ISSN (Online) 2456 -1304
International Journal of Science, Engineering and Management (IJSEM)
Vol 11, Issue 3, March 2024



Graph 4: IgG isotype in serum



Graph 5: NDV specific IgM + IgA isotype

Treatment: cells were grown, and they were treated with the antibody to check for protection against the virus. Vero cell line was revived and after 90% confluency, the cell line was further passaged with a split ratio of 1:3. The three flasks were labelled as control, Antibody + virus, Virus. The flasks were supplemented with fresh culture media and 200 µL of the virus was added. In the flask with the antibody and virus, 200 µL of the virus sample was mixed with 200 µL of purified monoclonal antibody in an Eppendorf tube. The mixture was incubated at RT for 1 hr. All the contents from the vial were transferred to the flask. The flasks were incubated for 4 days at 37°C in a 5% CO_2 incubator with 6% - 7% humidity.

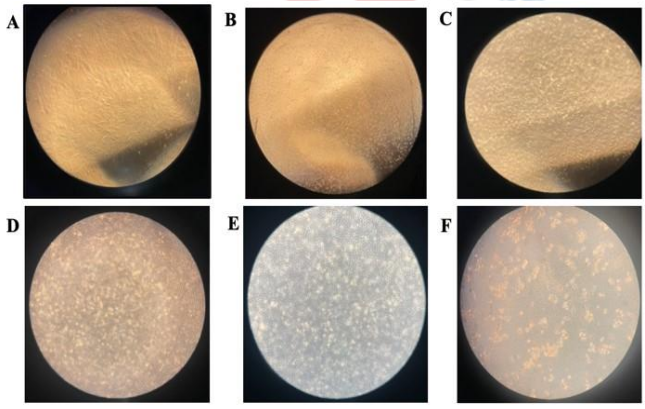


Figure 5: A-C: Vero cells grown in the flask before infection and treatment. D-F: After infection (F) and after treating the infected cells with the produced mAbs (E). A, D is the control. The image depicts protection against infection.

III. RESULTS AND DISCUSSION

Viral Pathogenicity: Under sterile conditions, the NDV was inoculated into the allantoic cavity of the 10 day old embryo. The allantoic fluid of the embryo was collected and HA was done. Rapid HA results were seen after a few minutes. Thus, to titrate the virus the microplate HA showed a titer of 256 HAU/0.1 mL.

Confirmatory assays for inactivation: The two methods to confirm the inactivation was followed. After the virus was inactivated using 0.2% formaldehyde, cellular assays were carried out. The inactivation of the virus was confirmed as the test to titrate the virus showed no change. The 12- well seeding also showed no morphological changes.

Antibody detection: After immunizing the mice with the first dose, 7 days later the serum of the mice was tested for the presence of antibodies by HAI assay. There were no antibodies formed, the titer was 0. During the course of immunization, various antibodies were formed at different levels in different groups. On day 20 both the diluted and undiluted mice groups showed a titer value ranging between 10-15. As the number of doses increased; the formation of antibodies also increased although the groups showed different levels at different time periods.

Monoclonal Hybridomas: Hybridomas were generated after the fusion of myeloma cells with antibody producing B cells,. The produced hybridomas were selected using HAT medium and each antigen that is the NDV was tested using HAI assay and ELISA for their specific antibody.

NDV specific antibody isotype: The monoclonal antibodies produced was purified using the protein A based spin column (affinity chromatography). The OD of the eluted fractions was measured at 450nm.

Protective efficiency: The generated monoclonal antibodies were tested for their protectiveness against cells that were infected with NDV. Upon receiving treatment with the monoclonal antibodies the cells retained their original shape as compared to the control, while the cells infected with virus had shrunk. Therefore, the monoclonal antibodies generated are very efficient in protecting the cells from infection.

IV. DISCUSSION

Monoclonal antibodies (mAbs) are an immensely important reagent for diagnostics and therapeutics. Generation of mAbs using hybridoma technology is relatively easier, convenient, straightforward and provides with an unlimited source of antibody producing cells. NDV was needed to be inactivated to prevent any possible mortality in the immunized animals hence, the NDV stock was inactivated using 0.2% formaldehyde.

Hemagglutinin assay showed that the inactivated virus particles contained the hemagglutinin which is an immunogenic NDV protein and presence of intact hemagglutinin on the inactivated virus particles is important from immunization as it is highly immunogenic and also it is

present on the external surface of the virus. Thus, the antibody generated against it will be useful from diagnostic and therapeutic point of view. Effective inactivation of the virus was also confirmed by growing the viruses on cultured Vero cells.

Generally, within a few weeks of immunisation, most of these plasma cells tend to accumulate in the spleen; therefore, splenocytes serve as the major source of B cells specific to desired antigen. Although a number of myeloma cells are available, the choice of it depends on compatibility with the immunized animal. The myeloma cell line SP2/0 was derived from BALB/c mice and does not produce any immunoglobulin making differentiation between the antibody from the myeloma and fused B cells affluent.

Affinity matured antibodies show stronger binding to its target antigen. As IgG antibodies are formed as a result of germinal centre reaction and isotype switching, they tend to be affinity matured. Also being the smallest antibody isotype, IgG antibodies are the preferred class from diagnostic and therapeutic point of view.

Using ELISA, elute containing purified IgG, was tested for the presence of NDV-specific IgG. ELISA results did show the presence of NDV- specific IgG antibody in the elutes.

Furthermore, IgG antibodies purified from hybridoma culture supernatant was tested for its protection and neutralisation efficiency. When tested on cultured Vero cells, the NDV preparation incubated for an hour with purified IgG did not cause any damage to the Vero cells confirming that the hybridoma generated through this work really bound to the virus and prevented its entry into Vero cells and thus, inhibited its propagation.

Eventually the clones for the best diagnostic antibody can be selected and further used for the development of rapid antigen detection kits.

V. CONCLUSION

Despite the fact that ND has been identified since 1926, it is still endemic in many poor nations and spreads throughout several bird species. It is possible that the respiratory system, digestive system, conjunctiva, injured skin, and cloacal mucosa all contribute to the rapid spread of NDV. The disease is characterised by high fever, diarrhoea, neurological dysfunction, and bleeding from the mucosa and serosa. For good prevention and successful control of NDV, there is an immediate need to develop quick detection systems and efficient medications.

With increasing technology of mAb production, hybridoma technology is the preferred way because of its inherent ability to continuously secrete antibodies. MAbs generated against NDV are essential for the identification of samples with the specific antigen collected by both infected hens who have died and those that are still alive. They may also be used to identify NDV antigen in chickens who attained the disease through the environment, which is extremely beneficial for histopathological investigations of NDV in infected persons.

The study clinches that serum from the mice showed the highest antibody concentration containing highest levels of IgG isotypes followed by IgM and IgA. Purified mAbs were then tested against cells infected with NDV and the cells survived, without any change in morphological characteristics.

Therefore, the development of mAbs by hybridoma technology against NDV would be a promising source of protection against the deadly disease. Developing the rapid detection antigen kit with the NDV specific antibody would help stop the spread of the disease worldwide reducing the loss to the poultry industry.

REFERENCES

[1] Astana Nyoman. (2016). *Immunological Detection of Newcastle Disease Viral Antigen in the Naturally Infected Chickens by Monoclonal Antibodies against Fusion2 Protein*.

[2] Barteling, S. J., & Woortmeyer, R. (1984). Formaldehyde inactivation of foot-andmouth disease virus. Conditions for the preparation of safe vaccine. *Archives of Virology*, *80*(2– 3), 103–117. https://doi.org/10.1007/BF01310652

[3] Burman, B., Pesci, G., & Zamarin, D. (2020). Newcastle Disease Virus at the Forefront of Cancer Immunotherapy. *Cancers*, *12*(12), 3552. https://doi.org/10.3390/cancers12123552

[4] Choi, K.-S., Lee, E.-K., Jeon, W.-J., & Kwon, J.-H. (2010a). Antigenic and immunogenic investigation of the virulence motif of the Newcastle disease virus fusion protein. *Journal of Veterinary Science*, *11*(3), 205. https://doi.org/10.4142/jvs.2010.11.3.205

[5] Choi, K.-S., Lee, E.-K., Jeon, W.-J., & Kwon, J.-H. (2010b). Antigenic and immunogenic investigation of the virulence motif of the Newcastle disease virus fusion protein. *Journal of Veterinary Science*, *11*(3), 205. https://doi.org/10.4142/jvs.2010.11.3.205

[6] DeFranco, A. L. (2016). The germinal center antibody response in health and disease. *F1000Research*, *5*, 999. https://doi.org/10.12688/f1000research.77 17.1

[7] Dimitrov, K. M., Abolnik, C., Afonso, C. L., Albina, E., Bahl, J., Berg, M., Briand, F.-X., Brown, I. H., Choi, K.-S., Chvala, I., Diel, D. G., Durr, P. A., Ferreira, H. L., Fusaro, A., Gil, P., Goujgoulova, G. V., Grund, C., Hicks, J. T., Joannis, T. M., ... Wong, F. Y. K. (2019). Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infection, Genetics and Evolution*, *74*, 103917. https://doi.org/10.1016/j.meegid.2019.103917

[8] Ghaniei A, & Moohammadzadeh n. (2012). Detection of Newcastle disease virus antibodies in serum of broiler chickens of Iran. *Journal of Animal and Poultry Sciences* .

[9] Goldhaft, T. M. (1980). Guest Editorial: Historical Note on the Origin of the LaSota Strain of Newcastle Disease Virus. *Avian Diseases*, *24*(2), 297. https://doi.org/10.2307/1589696

[10] Grodzki, A. C., & Berenstein, E. (2010). *Antibody Purification: Affinity Chromatography – Protein A and Protein G Sepharose* (pp. 33–41). https://doi.org/10.1007/978-1-59745- 3240_5

[11] Holzlöhner, P., & Hanack, K. (2017). Generation of Murine Monoclonal Antibodies by Hybridoma Technology. *Journal*

of Visualized Experiments, *119*. https://doi.org/10.3791/54832

[12] Iorio, R. M., Syddall, R. J., Sheehan, J. P., Bratt, M. A., Glickman, R. L., & Riel, A. M. (1991). Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. *Journal of Virology*, *65*(9), 4999–5006. https://doi.org/10.1128/jvi.65.9.4999- 5006.1991

[13] Mansour, F. (2016). Isolation of Newcastle disease virus (NDV) in embryonated chicken eggs. *Basrah Journal of Veterinary Researc*.

[14] Kaufmann, L., Syedbasha, M., Vogt, D., Hollenstein, Y., Hartmann, J., Linnik, J. E., & Egli, A. (2017). An Optimized Hemagglutination Inhibition (HI) Assay to Quantify Influenza- specific Antibody Titers. *Journal of Visualized Experiments*, *130*. https://doi.org/10.3791/55833.

[15] Le, V. P., Trinh, T. B. N., Nguyen, V. T., Nguyen, T. L., & Nuanualsuwan, S. (2022). Inactivation rate of African swine fever virus by a formaldehyde-based product. *Journal of Animal Science*, *100*(10). https://doi.org/10.1093/jas/skac248.

[16] .Pandey Shivanand. (2010). *Hybridoma technology for production of monoclonal antibodies*.

[17] Parray, H. A., Shukla, S., Samal, S., Shrivastava, T., Ahmed, S., Sharma, C., & Kumar, R. (2020a). Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives. *International Immunopharmacology*, *85*, 106639. https://doi.org/10.1016/j.intimp.2020.06639

[18] Parray, H. A., Shukla, S., Samal, S., Shrivastava, T., Ahmed, S., Sharma, C., & Kumar, R. (2020b). Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives. *International Immunopharmacology*, *85*, 106639. https://doi.org/10.1016/j.intimp.2020.106

[19] Zhang, L., Li, Z., Jin, H., Hu, X., & Su, J. (2018). Development and application of a monoclonal antibody-based blocking ELISA for detection of antibodies to Tembusu virus in multiple poultry species. *BMC Veterinary Research*, *14*(1), 201. https://doi.org/10.1186/s12917-018 1537-6

[20] Yamamoto, Y., Shimizu, S., Hirota, J., Kurokawa, A., & Mase, M. (2019). Development of a Monoclonal Antibodybased Immunohistochemical Method for Detecting Newcastle Disease Virus. *Japan Agricultural Research Quarterly: JARQ*, *53*(2),135–141. https://doi.org/10.6090/jarq.53.135

[21] Yang, F., Li, Y., Jin, X., Xu, Q., Cheng, F., & Wang, X. (2020). Immunosensor-based rapid quantitative detection of Newcastle disease virus antibodies using innovative gold immunochromatographic assay. *Journal of Applied Microbiology*, *129*(6), 1751–1757. https://doi.org/10.1111/jam.14688