Cytokine immune response following vaccination against Fowl Pox disease in Specific Pathogen Free chickens

Mohammad Reza Azizi [1*], Ismail Asli [1,2]

1- Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
2- Brucellosis Vaccine Production Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Exxtension Organization, Karaj, Iran.

Abstract

Fowlpox is an infectious disease with a relatively slow spread in all ages of poultry and is characterized by skin lesions in dry form or diphtheria-like lesions in the mouth in wet form. The disease causes slow growth and reduced egg production. This disease is one of the oldest known poultry diseases. Its virulence first was shown in 1902 by Marks & Sticher. The disease is widespread in all parts of the world and birds of any age, race and gender are susceptible to this virus and are also relatively sensitive. The present study was conducted to investigate Th1 and Th2 cells, respectively, cytokines IFN-γ and IL-4 in the culture medium supernatant of peripheral blood mononuclear cells stimulated with ConA mitogen in vaccinated and control chickens. This study included three groups of 40 21-day-old specific pathogen free chicks. One group was inoculated with PBS and served as a negative control, and the other two groups were vaccinated with Razi institute fowl pox vaccine and a commercial fowl pox vaccine. The injection of vaccine was done in the wing. All chicks were kept for 5 weeks after inoculation. Blood samples of each group were collected weekly up to 5th weeks after vaccination. Peripheral blood mononuclear cells (PBMC) were isolated from each blood sample using centrifuge ficoll-hippopotamus. The concentration of inflammatory cytokines (IFN-γ) and anti-inflammatory (IL-4) in peripheral blood mononuclear cells was measured using ELISA method. Statistical analysis and quantity ($P < 0.05$) were used to evaluate the significant difference between the groups. The results of this study showed that 7 days after vaccination, 90 to 100% of the vaccinated birds had swelling at the injection site. The ratio of the concentration of IFN-γ to IL-4 in supernatant was higher in the cell culture of vaccinated chicks than in the control group. The study states that induction of enhanced immune responses after fowl pox vaccination is mainly due to the Th1-lymphocytes response.

Keywords: fowl Pox vaccine, T lymphocytes, SPF chickens, PBMC

Introduction:

Fowl pox is a common disease in unvaccinated backyard chickens. The disease is usually self-controlled. The initial lesions of this disease starts as a white color blister and appears on the shoulder, and other areas of the skin In some rare cases, lesions can be seen on the body, legs and even softer parts of the beak. These blisters turn into a dark scab and take about three weeks to heal and go away. Chicken pox lesions in the mouth and throat of infected birds can cause breathing problems and even death In order to control the disease, vaccination of birds is recommended, and mosquito population management can also help in prevention of the disease. (1).The virus belongs to the genus Avipoxvirus in the family Poxviridae . The viral genome of the fowlpox virus is classified into three temporal classes: immediate, early, and late. Its large (~ 300 kb), linear double-stranded DNA genome has a hairpin loop at each end and encodes more than 200 genes, including those for DNA polymerase, NTPaseI, uracil glycosylase, and thymidine kinase. Significant variations between field and vaccine strains of the fowlpox virus have been shown by molecular analysis.The virus attaches itself to glycosaminoglycan receptors on the surface of the host cell to gain access.

Avian poxviruses have the ability to replicate in the cytoplasm, similar to other poxviruses. Unlike many other viruses, avian poxviruses can form extracellular enveloped viruses by budding mature virions directly through the plasma membrane (2)

The pathogenic mechanisms of fowlpox virus involve its resistance to the normal environment . This allows the virus to survive for long periods of time in dried scabs. The presence of photolyase and A-type inclusion body protein genes in the genome of fowlpox virus seems to provide protection against environmental challenges. The incubation period of fowlpox in chickens and turkeys typically ranges from 4 to 10 days (3). there have been reports of the virus infecting other bird species such as ducks, geese, pheasants, quail, canaries, and hawks. Mammals, on the other hand, are not susceptible to natural infection by the fowlpox virus or any other avipoxviruses. The virus is typically transmitted through contact with the skin abrasions of infected birds. Shed skin lesions (scabs) from recovering birds in poultry houses can serve as a source of aerosol exposure for susceptible birds. Additionally, mosquitoes and other biting insects may act as mechanical vectors for transmission. In multiple-age poultry complexes, the virus tends to persist for extended periods due to its slow spread and the presence of susceptible birds (4,5).

Fowlpox manifests in diverse clinical forms. In chickens and turkeys, it is characterized by the presence of proliferative lesions on the skin, which eventually develop into thick scabs, known as the cutaneous form. Additionally, lesions can also be found in the upper gastrointestinal and respiratory tracts, referred to as the diphtheritic form. In more severe cases, virulent strains of the virus can cause lesions in the internal organs, resulting in the systemic form. Localization of lesions around the nostrils can lead to nasal discharge, while cutaneous lesions on the eyelids may result in the complete closure of one or both eyes.
Caseous patches that strongly adhere to the mucosa of the mouth and larynx, or proliferative masses, may form in the diphtheritic form. Mouth lesions can disrupt feeding, while tracheal lesions can lead to breathing difficulties. It is crucial to distinguish laryngeal and tracheal lesions in chickens from those caused by infectious laryngotracheitis. A single bird may experience more than one form of the disease, such as cutaneous, diphtheritic, or systemic. The disease often progresses slowly within a flock, lasting between 2 to 8 weeks. Severe infection in a layer flock can lead to reduced egg production (6).To diagnose cutaneous fowlpox infections, microscopic examination of affected tissues stained with H&E is commonly employed. In addition fluorescent antibody and immunohistochemical methods are used. To isolate the fowlpox virus, it can be inoculated into the chorioallantoic membrane of developing chicken embryos, susceptible birds, or cell cultures of avian origin. Among these options, chicken embryos from a specific pathogen-free (SPF) flock, aged between 9 and 12 days, are the preferred and convenient host for virus isolation (7).
Given that most fowlpox outbreaks in previously vaccinated chickens are caused by strains harboring a genome containing full-length REV, the use of REV envelope-specific primers to ascertain the presence of full-length REV is beneficial in such instances.

Both naturally infected and vaccinated birds elicit humoral and cell-mediated immune responses. The humoral immune response can be assessed through ELISA, agar gel precipitation (AGP), or virus neutralization tests. While AGP is a straightforward and convenient test, its sensitivity is relatively lower when compared to ELISA (8).

There is no specific effective treatment for birds infected with fowlpox virus; therefore, prevention is key. Disease control is best accomplished by vaccination.In regions where fowlpox is prevalent, it is recommended to vaccinate chickens and turkeys with a live-embryo- or cell-culture-propagated virus vaccine. The most commonly used vaccines are live, attenuated fowlpox virus and pigeonpox virus isolates with high immunogenicity and low pathogenicity.

In areas with a high risk of infection, it is advisable to administer a live, attenuated virus vaccine of cell-culture origin within the first few weeks after hatching, followed by revaccination at 12–16 weeks of age.

The timing of vaccinations should be based on the health of the birds, level of exposure, and type of operation. Since the infection spreads slowly, vaccination can help limit the spread within affected flocks if given when less than 20% of the birds show lesions (9).

Passive immunity might hinder the replication of the vaccine virus; therefore, birds from recently vaccinated or infected flocks should only be vaccinated once passive immunity has waned.
After vaccination, birds should be monitored for swelling and scab formation at the vaccination site one week later. The absence of a vaccine take indicates a lack of vaccine potency, passive or acquired immunity, or improper vaccination technique. In such cases, revaccination with a different vaccine lot number may be necessary (9).
IFN-γ, also known as type II interferon, is a cytokine that plays a vital role in the immune response to viral, some bacterial, and protozoan infections. It activates macrophages (a type of immune cell) and helps increase the expression of class II molecules that are essential for immune recognition. Abnormal levels of IFN-γ are linked to various autoimmune and inflammatory diseases. Its importance in the immune system comes from its ability to directly inhibit viral replication and enhance immune responses. IFNG is mainly produced by natural killer (NK) cells and natural killer T (NKT) cells during the initial immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) cells as the immune response becomes more specific to the antigen. Type II IFN levels go up when interleukin cytokines like IL-12, IL-15, and IL-18 are produced, along with type I interferons (IFN-α and IFN-β). On the flip side, IL-4, IL-10, transforming growth factor-beta (TGF-β), and glucocorticoids are known to lower type II IFN levels. Interleukin 4 (IL4, IL-4) is a cytokine that helps turn naive helper T cells (Th0 cells) into Th2 cells. When Th2 cells get activated by IL-4, they go on to produce more IL-4 in a kind of positive feedback cycle. IL-4 mainly comes from mast cells, Th2 cells, eosinophils, and basophils. Interleukin 4 plays a lot of important roles in the body. It helps activated B cells and T cells multiply and also aids in turning B cells into plasma cells. It's a crucial player in both humoral and adaptive immunity. IL-4 encourages B cells to switch to producing IgE and boosts the production of MHC class II. On the flip side, IL-4 reduces the creation of Th1 cells, macrophages, IFNγ, and dendritic cells IL-12. IL-4 and IL-10 work as potent inhibitors of Th1 effector (10)
Defining chicken cytokine response to fowl pox vaccination Razi s vaccine and another imported fowl pox vaccine were used in this experiment and level of cytokine expressions was assessed by ELISA in the PBMC cell culture supernatant following stimulation with Concovalin A
Materials and Methods:
120 SPF chicks in third week of age were randomly divided into three groups, each of 40. The first group served as the target population for Razi ´s vaccine, the second group was vaccinated with an imported vaccine. The third group, was inoculated the same volume with Phosphate Buffered Saline as a non-vaccinated control group. All vaccinated chickens, as well as control group, were kept up to the fifth weeks after vaccination after five days PI (post inoculation), evidence of "Take" was observed at the inoculation site insuring the accuracy of the vaccination. (Figure-1)

Figure 1: Take is visible after the injection of the vaccine

Blood samples

Blood samples were taken from five chicks per group weekly through 5 weeks after vaccination, 1 cc blood from the heart and under sterile conditions mixed with EDTA then all samples in each group were pooled.

Ficoll–Hypaque density gradient centrifugation

5 cc blood+EDTA was poured into a falcon tube gently, followed by addition of 5 cc PBS. In a separate sterilized falcon tube, 3 cc ficoll with a density of 1.077 g/ml was poured. Gently and slowly, 10 cc blood mixed with PBS were poured onto the ficoll solution ensuring that the ficoll settled at the bottom of the tube, and the 10 cc blood layer stays on top.

The tubes were then subjected to centrifugation in low temperature of 8 °C in speed 400 g for 20 minutes. After centrifugation, four distinct layers were observed: plasma, PBMC, ficoll, and blood cells respectively from top to the bottom. The PBMC layer was carefully extracted. Once the PBMC was extracted, it was transferred to flask tubes and the volume was adjusted to 10 cc by adding PBS. (Figure 2.)

The samples were then subjected to centrifugation at 100 g for 10 minutes, followed by mixing with a vortex. This step was repeated, and the supernatant containing PBS was discarded. Finally, the 2 cc precipitate was mixed with 3% PBS/BSA solution.

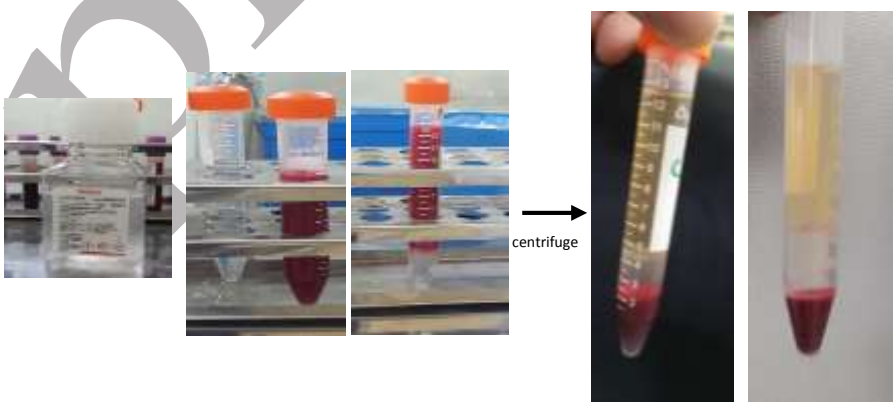

Figure 2: Preparing a PBMC with a Ficoll. By creating a 4-layer centrifuge, the image is created in accordance with the image. The first layer (from above) contains the plasma. The second layer contains PBMC cells. The third layer is the ficoll, and the fourth layer (bottom and red) contains red blood cells and multi-nucleated cells.

Culture of PBMC cells and stimulation of cells with ConA mitogen

The collected PBMC cells were washed twice with RPMI 1640 culture medium without antibiotics. The number of viable cells (viability) was determined by staining the cells with Trypan Blue. 2cc containing 10^7 line PBMC cells in RPMI 1640 culture medium inclusive of 10% fetal bovine serum (FCS) were cultured in each well of a 12-well microplates. Plates were incubated for 24 hours in 37° C with 5% CO2.

200 microliters ConA at a concentration of 10 micrograms/ml was added to each well then cells were incubated for 3 more days. After three days, the supernatant of each well was separated by centrifugation at 300 g for 5 minutes. This liquid containing secreted cytokine was kept in a freezer at -20°C until the time of cytokine measurement.

Measurement of cytokines:

The amount of cytokines produced in the supernatent isolated from the culture medium of PBMC cells after stimulation with ConA mitogen was measured by ELISA test (Biosource kit).

In summary, Samples and standards (in different dilutions) were added in separate wells of the plate in duplicate at a volume equal to 100 µl. After 2 hours of incubation at laboratory temperature, the monoclonal antibody against a specific cytokine (interferon gamma or interleukin 4) was added to each well in a volume equal to 100 µl. After the incubation of the plates (for 1 hour) 3 wash , 100 µl/well conjugated solution added. After 60 minutes at laboratory temperature and washing 5 times, 90 µl of the chromogen substrate was added to each well, after 30 minutes the reaction was stopped with 50 µl stop solution avoiding the light, and then OD of the plate was read in ELISA Reader in 450 nm. The amount of cytokines in each sample was calculated according to the standard curve.

Results:

In this study, IFN-γ and IL-4 cytokines induction capacity were measured in supernatant of the peripheral blood mononuclear cells culture medium which had been stimulated by ConA mitogen, in vaccinated and control chickens. The results showed that the IFN-γ concentration of chicken PBMC cell culture supernatant was higher compared to the control group (Figure 5-3). It was found that the level of Th1 cytokines such as IFN-γ increased in the vaccinated group. and it shows that the activity of Th1 cells becomes higher after vaccination(figure-2)

The concentration of IL-4, which is mainly secreted from Th2 cells, was also higher in the supernatant of the cell culture medium of the vaccinated group in the fourth and fifth weeks compared to the control group. and showed a significant difference (P>0.05).(figure-3)

There weren't any significant differences between group injected by Razi s vaccine and imported vaccine group

Induction of cytokine IFN-γ and IL-4 and in PMBC cells of chickens of the vaccinated group after stimulation with mitogen Con A compared to the control group are shown in table 1and 2 respectively

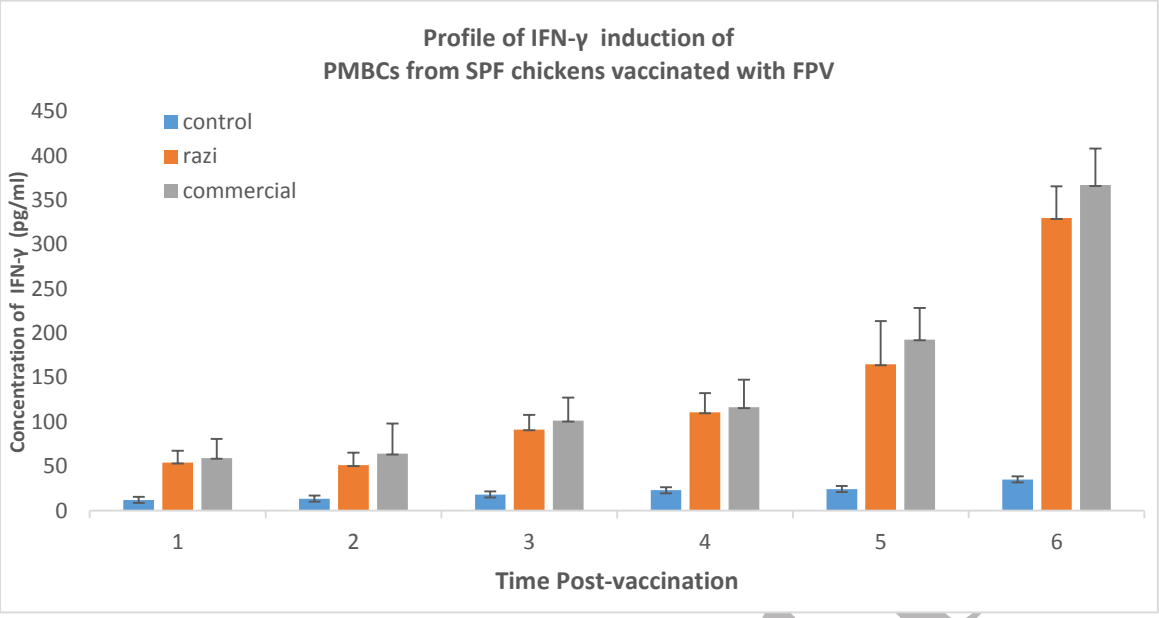

figure-2 : Cytokine induction of IFN-γ in PMBC cells of vaccinated chickens after stimulation with mitogenCon A compared to the control group.

.

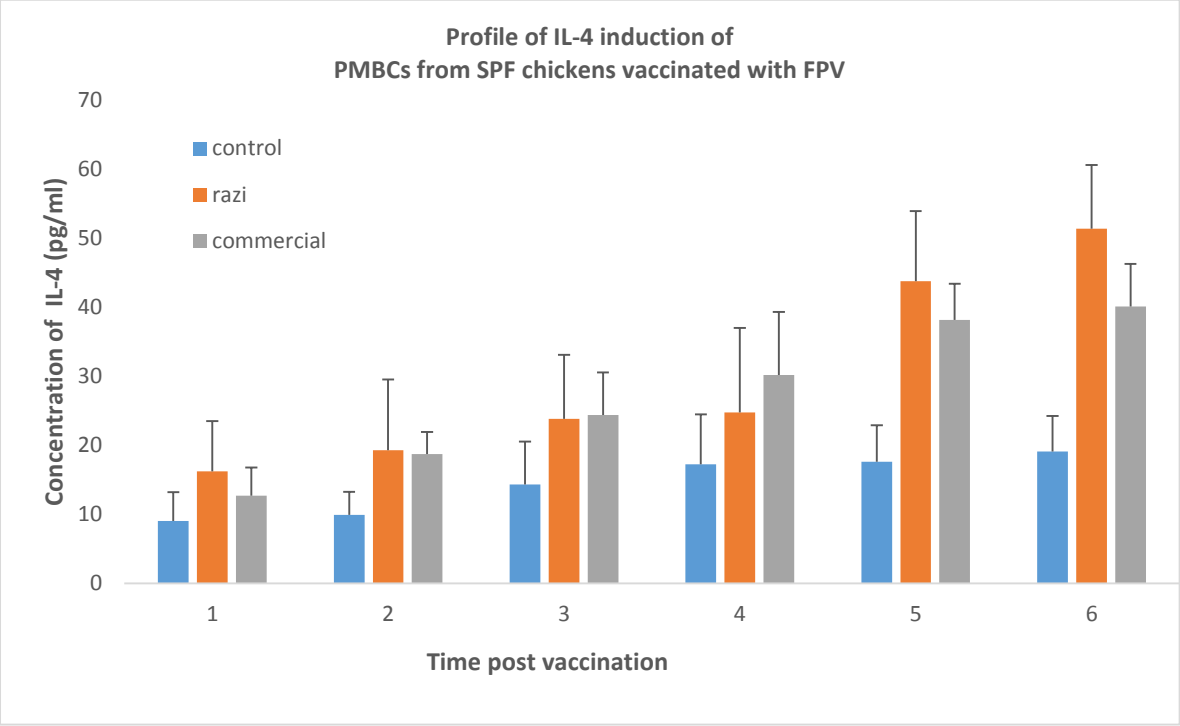

figure-3: IL-4 cytokine induction in PMBC cells of vaccinated chickens after stimulation with Con A mitogen compared to the control group

Table 1: Cytokine induction of IFN-γ in PMBC cells of vaccinated chickens after stimulation with mitogen Con A compared to the control group

Week	Control (pg/ml)	Razi FPV vaccine	Commercial FPV vaccine
week1	12.15 ± 6.83	54.33 ± 13.21	59.51 ± 21.34
week2	13.61 ± 4.21	51.25 ± 14.11	64.24 ± 34.18
week3	18.36 ± 3.14	91.62 ± 16.45*	101.42± 26.19*
week4	23.21 ± 10.16	110.98 ± 21.53*	116.71 ± 30.87*
week5	24.48 ± 13.11	164.97 ± 48.75*	192.97 ± 35.62*
week6 (Challenge)	35.23 ± 10.21	329.93 ± 35.68*	367.13 ± 41.23 *

*: ($P < 0.05$) Indicates a significant difference with the control group using paired t-test.

Table 2: Induction of cytokine IL-4 in PMBC cells of chickens of the vaccinated group after stimulation with mitogen Con A compared to the control group

Week	Control	Razi FPV vaccine	Commercial FPV vaccine
week1	9.04 ± 4.17	16.23 ± 7.26	12.67 ± 4.11
week2	9.91 ± 3.35	19.3 ± 10.23	18.72 ±3.21
week3	14.31 ± 6.21	23.84 ± 9.28	24.36 ±6.19
week4	17.25 ± 7.25	24.76 ± 12.22	30.19 ± 9.11
week5	17.62 ± 5.26	43.78 ± 10.16*	38.15 ± 5.27*
week6 (Challenge)	19.11 ± 5.13	51.39 ± 9.21*	40.11 ± 6.18*

*: ($P < 0.05$) The indicator of significant difference with the control group using paired t-test.

Discussion

Cytokines are proteins that are secreted from immune cells, which stimulate and regulate inflammatory and immune reactions. According to the specific biological effects of each cytokine, the deficiency or excess of each of them leads to disease and disorder in the immune system .Cytokines can be used as a therapeutic tool or as a target for specific antagonists, in a variety of immune and inflammatory diseases . A strong immune response that leads to the intense secretion of various cytokines can lead to inflammatory diseases the need to measure cytokines in their different behavior in body before and after taking drugs(11)., while on the other hand, cytokines such as INFγ and TNFα lead to the development or progression of inflammatory diseases IL-4 cytokine reduces chronic inflammatory diseases (12,13).

Cytokine secretion is commonly assessed using various assays, notably enzyme linked immunosorbent assay (ELISA), radioimmunoassay, and RT-PCR. Immunoassays like ELISA are favored for their simplicity, although they can struggle to detect low concentrations of cytokines. Additionally, mRNA levels measured by RT-PCR do not always correlate directly with protein levels, indicating the complexities in cytokine measurement (14-16).

In previous study by theses author flowcytometery evaluation was done on CD4+ and CD8+ cells, it was shown that cell mediated immunity is induced by fowl pox vaccination(17). In this study, it was

shown that the IFN-γ concentration of chicken PBMC cell culture supernatant was higher compared to the control group, which indicates that after vaccination against fowl pox the activity of Th1 cells becomes higher. Also, the concentration of IL-4, which is mainly secreted from Th2 cells, was also higher in the supernatant of the cell culture medium of the vaccinated group in the fourth and fifth weeks compared to the control group and showed a significant difference.it means that fowl pox vaccine leads to cytotoxic T cell immune response which is called cell mediated immunity. In 2014, a study on the detection of target antigens of B and T cells of domestic chickens was conducted by Roy et al. A high level of antibodies against the smallpox virus was observed in the tested birds. In this study, a significant increase in cell-dependent responses was observed in inoculated chickens, and the increase in T lymphocytes well indicated cell-dependent responses (18). In 1994, a study was conducted by Astrila, Vaino and Lasila regarding the central role of CD4+ T lymphocytes in birds. This study also showed that T lymphocytes play the main role against the viral agent (19). Jianing Wang and colleagues in 2006 used an indirect ELISA method to measure the specific antibody response of smallpox virus. The non-specific humoral response was evaluated by injecting two T-cell dependent antigens, sheep red cells and bovine serum albumin. There was no significant difference in antibody responses to smallpox virus between chickens infected with different isolates and strains of smallpox virus in both groups. In contrast, two weeks after inoculation the antibody responses were significantly lower. It could be interpreted that humoral immune response is not involved in immune response of chickens against fowl pox vaccine or virus they measured cellular immune responses by in vitro lymphocyte proliferation assays and using PHA-P skin tests and observed significantly enhanced cell immunity in chickens infected with both pox virus strains and vaccine strains. (20).

In 2016, a study by Bareda compared the immune response induced by a commercial fowlpox virus vaccine and the lesion at the inoculation site. In this research, performing ELISA on the antigen prepared from the cell culture medium, showed that there is a direct relationship between the values of ELISA and the presence of lesions after vaccination.(21)

In 2003, Puehler and his colleagues showed that in cells infected with fowl pox virus, the high activity of chicken interferon-gamma is destroyed by trypsin. In this study, using the laser ionization time analysis method of mass spectrometer, it was shown that the viral interferon gamma binding protein is encoded by the FPV 016 gene. Also, they showed that contrary to the previous findings regarding cellular interferon-gamma receptors and smallpox viruses that contain domains containing fibronectin type 3, in the birdpox virus, the interferon-gamma binding protein contains an immunoglobulin domain that does not interact with any cellular protein or No known virus has a similar homologous structure. In this study, an increase in immunoglobulins (which ultimately led to an increase in gamma interferon) was observed in the vaccinated groups (22).

In 2008, Holecheck et al. conducted a study of a recombinant smallpox virus to express the recombinant fungal interferon gamma gene and administered it to mice with the aim of providing concentrated levels of the cytokine to the tissue microenvironment, which showed a significant increase in the level of MHC class 1 expression(23)

The decreased mRNA expression of the IFNγ gene at late atages post-vaccination may be attributed to the transient nature of cytokine secretion, which occurs alongside the transcription of their respective genes. Various factors influence the immune response to vaccination, including maternal antibodies, antigen characteristics, adjuvants, host age, nutritional status, genetics, and stress. Understanding immune responses and vaccine efficacy is crucial for developing and optimizing vaccination strategies. (24)

Choosing the right method for quantifying cytokines is essential in immunological studies, as it influences the accuracy of assessing cellular responses to antigens. It's important to select assays

based on the specific laboratory capabilities and the nature of the immunological responses being investigated, as no single technique can be universally applied to all scenarios.

As a result, the present study showed that the immune system may be associated with an increase in cellular immunity, which is resulted in the proliferation of T lymphocyte cells and an increase in the ratio of the concentration of IFN- γ to IL-4 in groups of vaccinated chickens compared to the control group. This study states that induction of enhanced immune responses is mainly by Th1 type response.

Since the results of the study show that the cytokine test for measuring ChIFN-γ and IL-4 can be considered as a suitable method for measuring CMI in poultry,therefore The ELISA tests can have a high ability to measure the role of CMI in protection against poultry infectious diseases and following vaccination.

Acknowledgment The authors would like to extend their sincerest gratitude to our colleague at Razi institute for their invaluable contribution to all process step of this research.

Authors' Contribution

Study concept and design: I.A Acquisition of data: I.A and M.R. A Data analysis and interpretation: I.A and M.R. A Manuscript preparation: N.M. and M.R. A. Critical revision of the manuscript for important intellectual content: N.M.

Ethics

As no human or animal subjects were involved in this study, and the data were collected from previous studies conducted in Iran, ethical committee approval was not required.

Conflict of Interest

The authors declare that they have no conflict of interests.

Data Availability

The data that support the findings of this study are available on request from the corresponding autho

References

1- Nynke Teeninga, Annemieke J Willemze, Marieke Emonts, Inge M Appel. Acute illness following chicken pox: spleen infarction as a complication of varicella zoster infection. Ned Tijdschr Geneeskd. 2011;155(28): A2987.

2- Hye-Ryoung Kim , Il Jang, Hye-Soon Song, Si-Hyeon Kim, Hyeon-Su Kim and Yong-Kuk Kwon. Genetic Diversity of Fowlpox Virus and Putative Genes Involved in Its Pathogenicity. Microbiol Spectr. 2022 Oct 26;10(5):e0141522.

3- Gladys Yeo , Yifan Wang, Shin Min Chong, Mahathir Humaidi, Xiao Fang Lim, Diyar Mailepessov, Sharon Chan, Choon Beng How, Yueh Nuo Lin, Taoqi Huangfu, Charlene Judith Fernandez, Hapuarachchige Chanditha Hapuarachchi, Grace Yap. Characterization of Fowlpox virus in chickens and bird-biting mosquitoes: a molecular approach to investigating Avipoxvirus transmission. J Gen Virol. 2019 May;100(5):838-850

4- Afonso C.L., Tulman E.R., Lu Z., Zsak L., Kutish G.F., Rock D.L. Rock. The Genome of Fowlpox Virus. Journal of Virology. 2000 April. 74(8):3815-31

5-Miguel Matos, Ivana Bilic, Nicola Palmieri, Peter Mitsch, Franz Sommer, Jana Tvarogová, Dieter Liebhart, Michael Hess. Epidemic of cutaneous fowlpox in a naïve population of chickens and turkeys in Austria: Detailed phylogenetic analysis indicates co-evolution of fowlpox virus with reticuloendotheliosis virus. Transbound Emerg Dis. 2022 Sep;69(5):2913-2923.

6- Majid Gholami-Ahangaran, Noosha Zia-Jahromi, Abdolrasul Namjoo. Molecular detection of avian pox virus from nodular skin and mucosal fibrinonecrotic lesions of Iranian backyard poultry. Trop Anim Health Prod. 2014 Feb;46(2):349-53.

7- Amanda M MacDonald, Daniel J Gibson, John R Barta, Rebecca Poulson, Justin D Brown, Andrew B Allison, Nicole M Nemeth. Bayesian Phylogenetic Analysis of Avipoxviruses from North American Wild Birds Demonstrates New Insights into Host Specificity and Interspecies Transmission. Avian Dis. 2019 Sep 1;63(3):427-432.

8-Jianning Wang, Joanne Meers, Peter B Spradbrow, Wayne F Robinson. Evaluation of immune effects of fowlpox vaccine strains and field isolates. Vet Microbiol. 2006 Aug 25;116(1-3):106-19

9-Deoki N Tripathy. The impact of vaccines and the future of genetically modified poxvirus vaccines for poultry. Anim Health Res Rev. 2004 Dec;5(2):263-6.

10-**Production of interferon gamma and interleukin 17A in chicken T-cell subpopulations hallmarks the stimulation with live, irradiated and killed avian pathogenic *Escherichia coli*panelSina Bagheri [a], Surya Paudel [a 1], Viskam Wijewardana [b], Richard,Thiga Kangethe [b], G iovanni Cattoli [b], Michael Hess [a], Dieter Liebhart [a], Taniya Mitra .** Developmental & Comparative Immunology
Volume 133, August 2022, 104408

11- Patrick Blanco, A. Karolina Palucka, Virginia Pascual, and Jacques Banchereau, Dendritic cells and cytokines in human inflammatory and autoimmune diseases. Cytokine Growth Factor Rev. 2008 February; 19(1): 41–52.

12- Sonobe Y, Jin S, Wang J, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A. Chronological changes of CD4(+) and CD8(+) T cell subsets in the experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. Tohoku J Exp Med. 2007 Dec;213(4):329-39

13- Alberto Alonso-Ruiz, Jose Ignacio Pijoan Eukene, Ansuategui, Arantxa Urkaregi, Tumor necrosis factor alpha drugs in rheumatoid arthritis: systematic review and metaanalysis of efficacy and safety. BMC Musculoskeletal Disorders 2008, 9:52

14 **Ekerfelt, C., Ernerudh, J., & Jenmalm, M. C. (2002). Detection of spontaneous and antigen-induced human interleukin-4 responses in vitro: comparison of ELISPOT, a novel ELISA and real-time RT-PCR. Journal of Immunological Methods 260(1-2): 55–67.**
15- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif 25(4): 402–408. R

16-Roy.B, Joardar.SN, Samanta.I, Das.PK, Alam.SS, Nandi.S. Detection of T- and B-cell Target Antigens of Fowlpox Virus Isolated from Backyard Chickens in India. Vet Res. 2014 Oct 30; 45:107

17- M. R Azizi; E Asli; A. M Behroozikhah; B Khalesi.2021. Flow Cytometric Evaluation of CD4+ and CD8+ T-cell Immune Response in SPF Chickens Induced by Fowlpox Vaccine. Volume 76, Issue 3, September 2021, Pages 429-436

18-Schmidt MW, Houseman A, Ivanov AR, Wolf DA. (2007). Comparative proteomic and transcriptomic profiling of the fission yeast Schizosaccharomyces pombe. Molecular Systems Biology 3: 79.

19- Arstila TP, Vainio O, Lassila O. Central role of CD4+ T cells in avian immune response. Poult Sci. 1994 Jul;73(7):1019-26.

20- Jianning Wang, Joanne Meers, Peter B. Spradbrow, Wayne F. Robinsonc. Evaluation of immune effects of fowlpox vaccine strains and field isolates. Veterinary Microbiology. 2006: 116:106-119

21- **Barreda C. B. 2016. Relationship Between Values of Fowlpox ELISA and the Presence of "Takes" After Vaccination.** Avian Dis. 60(1):67-9. doi: 10.1637/11160-051615-ResNote.1.

22- Puehler F, Schwarz H, Waidner B, Kalinowski J, Kaspers B, Bereswill S, Staeheli P. An interferon-gamma-binding protein of novel structure encoded by the fowlpox virus. J Biol Chem. 2003 Feb 28;278(9):6905-11. Epub 2002 Dec 13

23- Susan A. Holechek , Karen L. Denzler , Michael C. Heck , Jill Schriewer , R. Mark Buller , Fatema A. Legrand , Paulo H. Verardi , Leslie A. Jones , Tilahun Yilma , Bertram L. Jacobs.2013. Use of a Recombinant Vaccinia Virus Expressing Interferon Gamma for Post-Exposure Protection against Vaccinia and Ectromelia Viruses. PLOS ONE | www.plosone.org 1 October 2013 | Volume 8 | Issue 10 | e77879

24-Joseph, L., Fink, L.M., Hauer-Jensen, M., 2002. Cytokines in coagulation and thrombosis: a preclinical and clinical review. Blood Coagul Fibrinolysis 13, 105-116.