Archives of Razi Institute, Vol. 76, No. 3 (2021) 429-436

DOI: 10.22092/ari.2020.343514.1508

Original Article

Flow Cytometric Evaluation of $CD4^+$ and $CD8^+$ T-cell Immune Response in SPF Chickens Induced by Fowlpox Vaccine

Azizi, M. R[1], Asli, E[1, 2 *], Behroozikhah, A. M[3], Khalesi, B[4]

1. Department of Microbiology, Faculty of Sciences, Islamic Azad University, Karaj Branch, Karaj, Iran
2. Department of Research & Development, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
3. Department of Brucella Vaccines Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
4. Department of Poultry Vaccines Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Received 4 July 2020; Accepted 5 September 2020
Corresponding Author: e.asli@rvsri.ac.ir

Abstract

Fowlpox (FP) is a viral disease that is widely distributed throughout the world. The disease has an economic impact on the poultry industry, and its prevalence has even been reported in vaccinated flocks. The present study used flow cytometry to evaluate the $CD4^+$ and $CD8^+$ T-cell immune response of chicks induced by FP vaccine. 120 specific pathogen-free (SPF) 21-day-old chicks were randomly divided into three groups of 40. One group was used as negative control with PBS inoculation, the other two groups were inoculated with the local fowlpox vaccine produced by Razi Institute and commercial FP vaccines, and they were kept for five weeks. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll–Hypaque density gradients and the percentages of $CD3^+$, $CD3^+$, $CD4^+$, and $CD3^+CD8^+$ T lymphocytes were analyzed with flow cytometry. Seven days post-immunization, a maximum (90-100%) swelling formation ("take") on the vaccination site was observed. The ratios of $CD4^+$ to $CD8^+$ T-lymphocytes in both vaccinated groups were significantly higher ($p < 0.05$) than the control group inoculated with PBS. The percentages of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T-lymphocytes were increased in chickens vaccinated with commercial and local FP vaccines. There were no significant differences between the groups receiving commercial and local fowl pox vaccines. The present study showed that protective immunity could be associated with increased cellular immune responses, which has been interpreted as enhancing T-cell proliferation and increasing $CD4^+$ to $CD8^+$ ratios through vaccination with the FP vaccine. This study further suggests that the induction of enhanced immune responses is due mainly to the Th1-type response.

Keywords: fowlpox vaccine, $CD4^+/CD8^+$ T lymphocytes, SPF chickens, flow cytometry

Évaluation Cytométrique en flux de la Réponse Immunitaire des Lymphocytes T $CD4^+$ et $CD8^+$ chez les Poulets SPF Induite par le Vaccin de la Variole

Résumé: La variole est une maladie virale largement répandue dans le monde. La maladie a un impact économique sur l'industrie avicole et sa prévalence a même été rapportée dans des troupeaux vaccinés. La présente étude a utilisé la cytométrie en flux pour évaluer la réponse immunitaire cellulaire des lymphocytes T $CD4^+$ et $CD8^+$ des poussins induite par le vaccin de la variole. 120 poussins de 21 jours exempts d'agents pathogènes spécifiques (SPF) ont été répartis au hasard en trois groupes de 40. Un groupe a été utilisé comme contrôle négatif avec une inoculation du PBS, les deux autres groupes ont été inoculés avec le vaccin

local contre la variole aviaire produit par l'Institute Razi et des vaccins commerciaux de la variole, et ils ont été conservés pendant cinq semaines. Les cellules mononuclées du sang périphérique (CMSP) ont été isolées à l'aide de gradients de densité Ficoll-Hypaque et les pourcentages de lymphocytes T $CD3^+$, $CD3^+CD4^+$ et $CD3^+CD8^+$ ont été analysés par cytométrie en flux. Sept jours après l'immunisation, une formation de gonflement maximale (90-100 %) ("prise") sur le site de vaccination a été observée. Les ratios de lymphocytes T $CD4^+$ à $CD8^+$ dans les deux groupes vaccinés étaient significativement plus élevés ($p<0.05$) que le groupe témoin inoculé avec du PBS. Les pourcentages de lymphocytes T $CD3^+$, $CD3^+CD4^+$ et $CD3^+CD8^+$ ont été augmentés chez les poulets vaccinés avec des vaccins commerciaux et locaux de la variole. Il n'y avait pas de différences significatives entre les groupes recevant des vaccins commerciaux et locaux contre la variole aviaire. La présente étude a montré que l'immunité protectrice pourrait être associée à une augmentation des réponses immunitaires cellulaires, qui a été interprétée comme une augmentation de la prolifération des lymphocytes T et une augmentation des rapports $CD4^+$ à $CD8^+$ grâce à la vaccination avec le vaccin de la variole. Cette étude suggère en outre que l'induction de réponses immunitaires améliorées est principalement due à la réponse de type Th1.
Mots-clés: vaccin contre la variole aviaire, lymphocytes T $CD4^+/CD8^+$, poulets SPF, cytométrie en flux

1. Introduction

Fowlpox (FP) is an ancient viral disease that was identified decades ago. It is derived from the ortho-pox virus and *Poxviridea* family (1). The disease spreads gradually on the skin of the birds, especially in the unfeathered areas of the head, neck, and legs. Lesion may also develop in the upper part of the respiratory tract that is known as the diphtheric form (2). Almost all bird species are susceptible to this disease, but it is most often found in domestic birds, especially chickens, cocks, and turkeys (3). It is seen in all age groups and has an economic impact on the poultry industry. Its prevalence has even been reported in vaccinated flocks (4, 5). Several strains of the virus that are highly interdependent but indissoluble are known to cause disease in birds, including poxviruses, turkey pox, canarypox and quailpox, sometimes with cross-strain immunity (6). To effectively control infectious diseases, a variety of vaccination programs can be implemented (7). The main role of vaccines is to control disease in chickens, and clearly, a better understanding of the mechanisms involved in protecting chickens is necessary so as to facilitate the development of new vaccines and improve existing vaccine regimens (8). When using a vaccine containing a live virus, a mild infection begins, which results in an immune response and the production of specific antibodies. In almost all provinces in Iran, fowlpox vaccinations are utilized to prevent disease outbreaks (9). There is a fowlpox vaccination program for laying, breeders, and parent flocks (10). The most used kind of vaccine in all parts of the world is a live attenuated one that provides active and powerful immunization against the disease (11). Cell-mediated immunity plays an important role in fowlpox vaccine-induced immunity in chicks, and techniques to assess T-cell responses are valuable tools in planning for controlling the disease (12). One of the most valuable tools for studying poultry immune systems is flow cytometry, because it has the unique ability to examine the expression of multiple antigens in each cell, thus distinguishing between T cells subsets (13). The present study was carried out using a multiple flow cytometry, which is more applicable in phenotypic assays. The cellular immune system in chicks is quite complex (14). The present study selected cell-mediated immune responses following application of the fowlpox vaccine as the disease model. Vaccination is known to prevent or reduce disease and is widely used in many countries (15). The FP vaccine stimulates the immune system, and T cells seem to play a critical role in protection and the clearance of the virus. (16). The current study used flow cytometry to evaluate the T cell responses in the peripheral blood following the immunization of SPF chickens with local and commercial FP vaccines.

2. Material and Methods

2.1. Experimental Chickens

120 SPF chickens were obtained from Razi Vaccine and Serum Research Institute and divided into three groups (21 days old, n=40 per group); one group served as the negative control (PBS) and the other two groups were inoculated with either the local FP vaccine or the commercial vaccine.

2.2. Immunization

The lyophilized FP (local) vaccine produced at Razi Vaccine and Serum Research Institute and the imported lyophilized FP commercial (Izovac Fowl-Pox, Live Attenuated Fowl Pox Virus, Italy) vaccine were used in this study. Each dose of vaccine titre was at least $10^{2.5}$EID50 embryo infective dose. All chickens were vaccinated by wing-web puncture with a double needle. All chickens were kept for 5 weeks following inoculation and monitored daily throughout the experiment for any abnormal behavior. Blood samples from four chickens in each group were collected in tubes containing EDTA (120 mg/mL) (Sigma Aldrich) at weeks 1, 2, 3, 4, and 5 after vaccination.

2.3. PBMCs Isolation

The blood samples collected from each group were diluted 1:1 with phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation according to the method of Dalgaard, Norup (17), using Ficoll–Hypaque density 1.077 g/ml (Sigma Aldrich). Briefly, 10 mL of diluted blood was carefully layered on top of Ficoll–Hypaque in a tube and then centrifuged for 20 minutes at 400 g, causing mononuclear fractioning by Ficoll–Hypaque density gradient separation. The cells in the interphase layer were aspirated and washed three times. The supernatant was removed and a pellet of PBMCs was collected. The cells were resuspended and then adjusted to a concentration of $1x10^7$ cells/ml with PBS for further assays.

2.4. Flow Cytometric Analysis of PBMCs

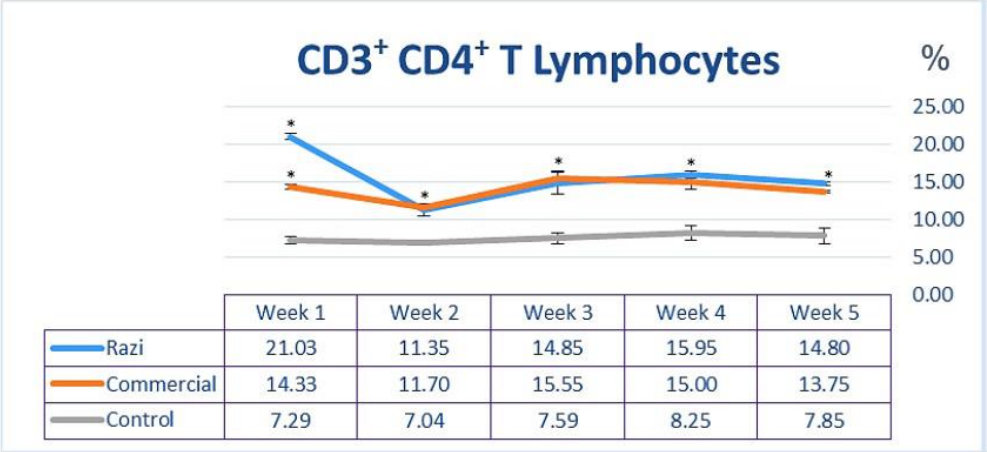
The percentage populations of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ lymphocytes in the PBMCs were analyzed using flow cytometry. Briefly, 50 µL of the PBMCs (1 x 10^6 cells) was incubated for 30 min at 4 °C in a dark place with 10 µL of mouse anti-chicken CD3-Spectral Red (SPRD) and 10 µL mouse anti-chicken CD4-R-phycoerythrin (R-PE), or 10 µL mouse anti-chicken CD8a-R-PE (Southern Biotech, Birmingham, AL), and 10 µL mouse IgG1-FITC, respectively. All flow cytometry analyses were performed on a Partec PAS equipped with a 488 nm blue laser and a 640 nm red laser. Data was analyzed with the help of FlowJo software. Statistical analysis was used to evaluate significant differences between the groups, and $p<0.05$ was considered statistically significant.

2.5. Statistical Analysis

All data is presented as mean ± SD, and the experimental groups were used. Statistical analysis with SARS v.6.0 software was used to evaluate significant differences between the groups, and $p<0.05$ was considered statistically significant.

3. Results

The results of this study showed a swelling and scab formation in vaccinated chickens after one week ("take") at the site of vaccination. A maximum sign "takes" at day 7 post-vaccination (90-100%) was observed. The absence of "take" indicated the lack of effectiveness of the vaccine or improper vaccination, and the birds showed no symptoms of the disease. Vaccinated chickens from all groups showed significantly higher percentage populations of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells than the control group (Figure 1 and Figure 2). Significant increases in CMI responses were observed in chickens inoculated with commercial and local Razi fowl pox vaccines as assessed by flow cytometry (Figure 3 and Figure 4). The levels of $CD4^+$ and $CD8^+$ lymphocyte populations in samples vaccinated with the Razi vaccine were not significantly different from the commercial ones, and in some cases even relatively tangible. $CD4^+$ expression was relatively higher in both the secretion and commercial samples than the expression of $CD8^+$ (Table 1).



	Week 1	Week 2	Week 3	Week 4	Week 5
Razi	21.03	11.35	14.85	15.95	14.80
Commercial	14.33	11.70	15.55	15.00	13.75
Control	7.29	7.04	7.59	8.25	7.85

Figure 1. Comparison of local Razi fowlpox vaccine and commercial samples. The population rates of $CD3^+CD4^+$ were increased. Statistically significant differences ($p<0.05$) are indicated by * compared with the control group.

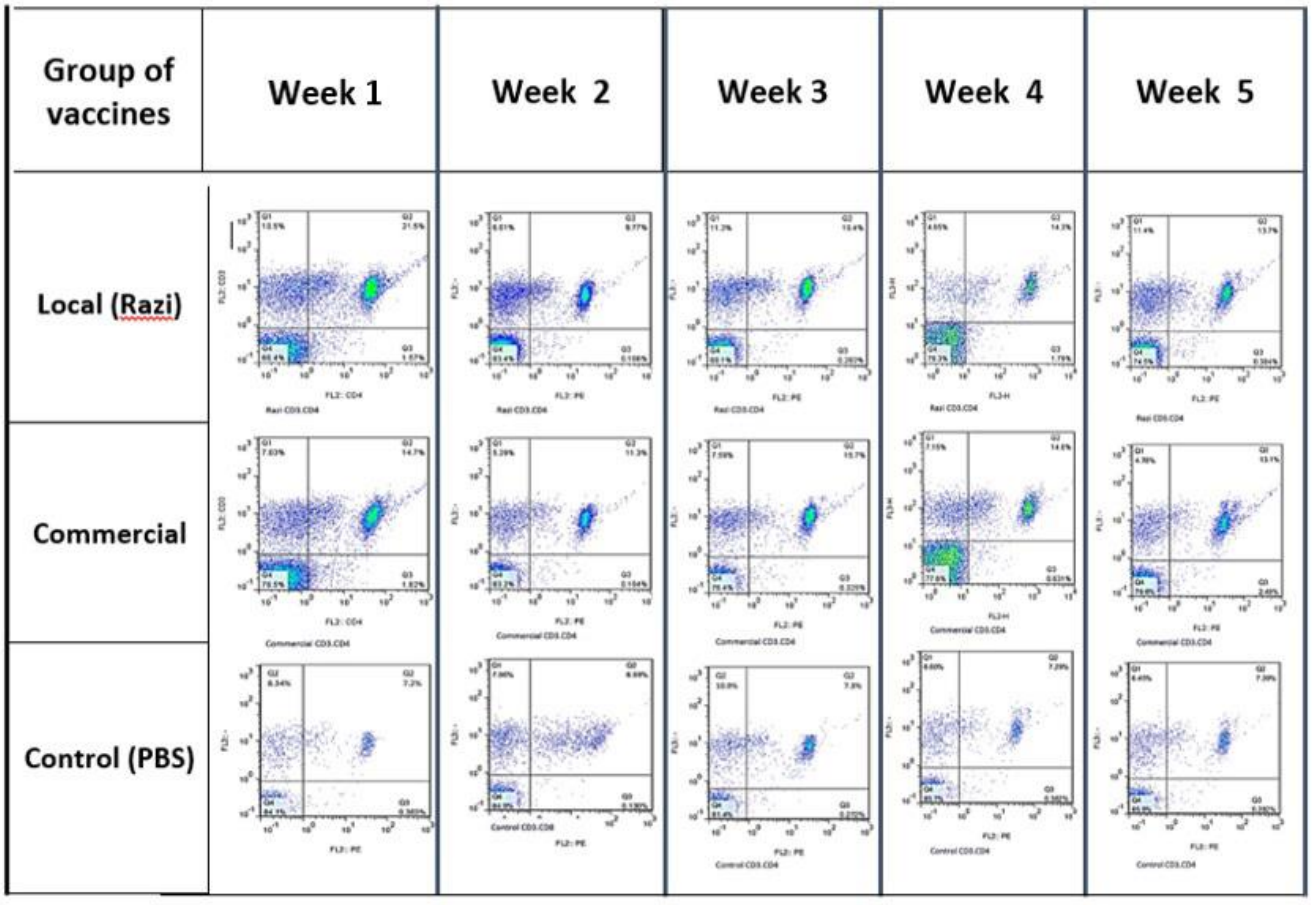


Figure 2. Typical flow cytometric pattern of $CD4^+$ cell expression in chickens vaccinated with local Razi fowlpox vaccine and commercial samples. Cells were gated using forward and side scatter to distinguish their physical properties and cellular contaminants. Percentages of $CD3^+CD4^+$ T cells among chicken PBMC were calculated from the upper right quadrant. The upper left quadrant represented $CD3^+$ $CD4^+$ T cells. Anti-chicken $CD3^+$ monoclonal antibodies were conjugated with SPRD and $CD4^+$ with Fluorescein R-Phycoerythrin (PE).

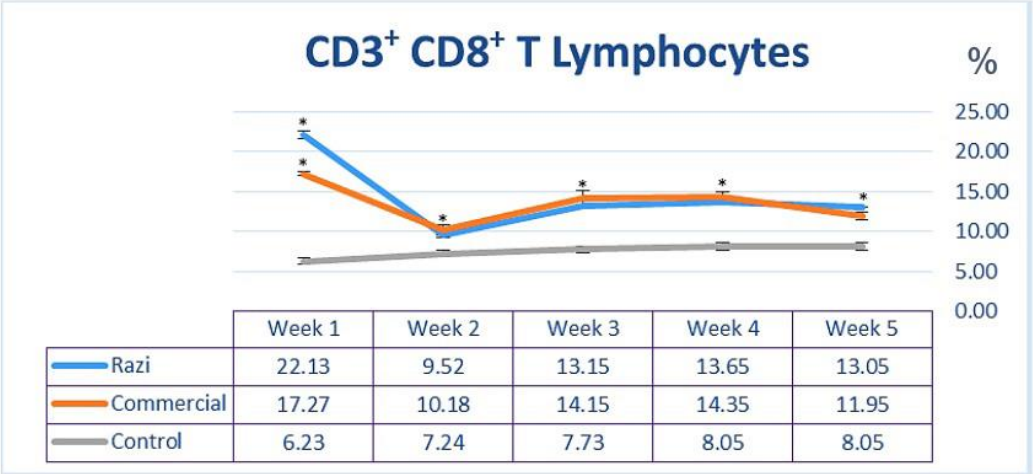


Figure 3. Comparison of local Razi fowlpox vaccine and commercial samples. The populations' rates of $CD3^{+}CD8^{+}$ were increased. Statistically significant differences ($p<0.05$) are indicated by * compared with the control group.

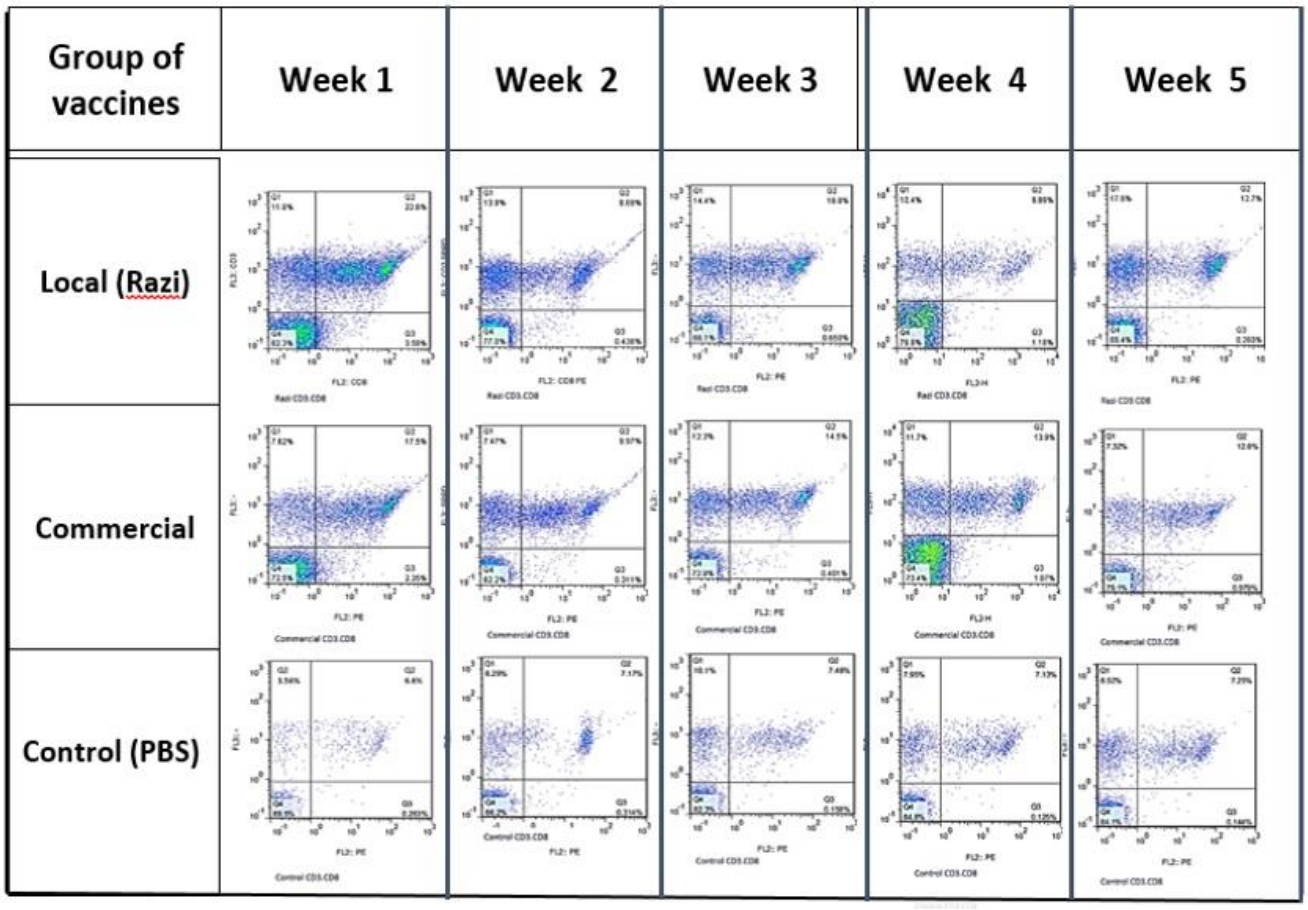


Figure 4. Typical flow cytometric pattern of $CD8^{+}$ cell expression in chickens vaccinated with local Razi fowlpox vaccine and commercial samples. Cells were gated using forward and side scatter to distinguish their physical properties and cellular contaminants. Percentages of $CD3^{+}CD8^{+}$ T cells among chicken PBMC were calculated from the upper right quadrant. The upper left quadrant represents $CD3^{+}$ $CD8^{+}$ T cells. Anti-chicken $CD3^{+}$ monoclonal antibodies were conjugated with SPRD and $CD8^{+}$ with Fluorescein R-Phycoerythrin (PE).

Table 1. Ratio of $CD4^+$ to $CD8^+$ T lymphocytes.

Vaccine Group		Week 1	Week 2	Week 3	Week 4	Week 5
Control	**$CD4^+$: $CD8^+$**	0.95 ± 0.01	1.17 ± 0.10	1.10 ± 0.09	1.12 ± 0.03	1.26 ± 0.16
Local	**$CD4^+$: $CD8^+$**	0.42 ± 0.03	1.06 ± 0.06	1.03 ± 0.03	1.00 ± 0.06	0.98 ± 0.01
Commercial	**$CD4^+$: $CD8^+$**	0.83 ± 0.01	1.14 ± 0.03	1.09 ± 0.01	1.05 ± 0.02	1.04 ± 0.02

$CD4^+$ and $CD8^+$ cell populations were obtained from flow cytometry analysis. Data was expressed as mean ± SD (n=3 experiments). The mean of $CD4^+$ and $CD8^+$ lymphocytes following the local FP vaccine and commercial sample compared with the control group.

In the first week, levels of $CD4^+$ and $CD8^+$ expression in chickens vaccinated with the Razi vaccine and the commercial vaccine were much higher than the control samples. In the second week, the expression of $CD4^+$ and $CD8^+$ lymphocytes in chickens vaccinated with the Razi fowlpox vaccine and the commercial vaccine against fowlpox was lower than the previous week. In the third week, the expression of $CD4^+$ and $CD8^+$ lymphocyte in samples vaccinated with Razi fowlpox vaccine was more than samples vaccinated with the commercial vaccine. In the fourth and fifth weeks, the expression levels of $CD4^+$ and $CD8^+$ lymphocytes were constant for the vaccinated groups.

Ratios of $CD4^+$ to $CD8^+$ T cells ($CD4^+/CD8^+$) obtained from flow cytometric analyses were calculated to determine the relative fluctuation of CD8 cells in comparison to $CD4^+$ cells. The control group had a higher $CD4^+$ to $CD8^+$ ratio than the vaccinated groups, indicating a smaller $CD8^+$ cell fraction present in circulating T lymphocytes. This was expected, as the unvaccinated group does not trigger $CD8^+$ cells, which respond to an antigen presented by Class I MHC molecules.

The lower ratios of $CD4^+$ to $CD8^+$ lymphocytes in both groups of commercial and local Razi fowlpox vaccinated groups were significantly higher ($p<0.05$) than the control group inoculated with PBS from the first week after vaccination (Table 1). The percentages of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T-lymphocytes were increased ($p<0.05$) in chickens vaccinated with commercial and local Razi fowlpox vaccines. There were no significant differences between both groups of vaccines. However, the ratios increased during the weeks, suggesting less $CD8^+$ cell populations or more $CD4^+$ cell populations were present in both vaccine groups.

4. Discussion

In poultry immune systems, the activation and proliferation of lymphocytes have important tasks in humoral and cellular immunological responses upon vaccination. Therefore, the current study evaluated the fowlpox vaccine in chickens and whether it could influence cellular immune responses. The results showed that T cells from fowlpox vaccinated chickens have proliferative responses. Levels of the T cell proliferative responses in the local and the commercial group were significantly higher than the control group, indicating that both local and commercial vaccines were able to stimulate proliferation of T-cells and the protection of chickens against disease challenges (18). It could be concluded that the protective effect may be due to enhanced cell-mediated immune response and increased $CD4^+$ to $CD8^+$ ratios. Flow-cytometric assay is an effective and efficient method for evaluating cell-mediated immune responses (19).

Th1 cytokines are molecules involved in immune responses. They are produced by mononuclear phagocytes and other cell types in response to viral infection and stimulate the proliferation of cytotoxic T-lymphocytes and NK cells in chickens (20). The T-lymphocyte proliferation responses resulted in cell-mediated immunity, preferably Th1 type lymphocyte (21). At one and five weeks after vaccination, peripheral $CD4^+$ and $CD8^+$ cell proliferation was found to be significantly higher in the Razi vaccine than in the commercial vaccine at both sampling times.

Flow cytometric assay has been emphasized as a very useful tool for the study of T cell biology in chickens (17). Using a flow cytometer, the $CD4^+/CD8^+$ ratios were calculated from the population of cells labeled with the fluorescent monoclonal antibodies of anti-$CD4^+$ or anti-$CD8^+$ (22). Ratios of $CD4^+CD8^+$ T lymphocytes determine the relative fluctuation of $CD8^+$ cells in comparison to $CD4^+$ cells. A higher ratio of $CD4^+CD8^+$ T lymphocytes reveals a smaller $CD8^+$ cell fraction present in circulating T cell populations. After the first week, the highest $CD4^+$ count was observed and eventually stabilized. The $CD8^+$ rate was not significantly different between the Razi and the commercial vaccine, and it was more relevant compared to the control group.

In comparing the local Razi vaccine with the commercial imported fowlpox vaccine, it was determined that the highest populations of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ were in the group receiving the Razi vaccine with statistically significant differences ($p<0.05$). Kannan, Geetha (23) found the highest $CD4^+$ and $CD8^+$ populations at day one as well as the lowest population at twenty weeks of age. $CD8^+$ cells were shown to be significantly higher than the $CD4^+$ cells. A significant difference was observed in the spleen at different ages in the ratio of $CD4^+$ to $CD8^+$ cells. The ratio of $CD4^+$ to $CD8^+$ T cells determines the relative fluctuation of $CD8^+$ cells compared to CD4+ cells. The ratio of $CD4^+$ to $CD8^+$ was shown to be higher in the control group than in the vaccinated groups. However, it is suggested that the future development of the flow cytometry method will result in the ability to measure the function of cell immunity in protecting against poultry diseases. It is also suggested that with a combination of a variety of T cell parameters including Th9 and Treg in poultry, this method is to be considered for identifying the valuable concepts of the safety mechanism. Moreover, evaluating cell immunity in chickens vaccinated on the first day against fowlpox, and measuring the immunity of different commercial vaccines by flow cytometry are other suggestions for future studies. Cytokine testing to measure ChIFN-γ and IL-4 can be considered as an appropriate method for measuring CMI in poultry (24). In conclusion, the present study showed that protection could be associated with increased cell-mediated immune response, which has been interpreted as an increase in T cell proliferation and enhanced $CD4^+$ to $CD8^+$ ratios following fowlpox vaccination. The present study showed the higher immunity and effectiveness of the local fowlpox vaccine in comparison with the commercial (Izovac Fowl-Pox) vaccine. This study showed the induction of cell-mediated immunity in the vaccinated chickens by the proliferation of specific antigens of CD4 and CD8 cells. In addition, the results showed that the cellular immune system is more preferably induced by increasing the Th1 type rather than Th2 type lymphocytes.

Authors' Contribution

Study concept and design: E. A.
Acquisition of data: M. R. A., and E. A.
Analysis and interpretation of data: M. R. A., E. A., A. M. B. and B. Kh.
Drafting of the manuscript: M. R. A., and E. A.
Critical revision of the manuscript for important intellectual content: M. R. A., E. A., A. M. B. and B. Kh.
Statistical analysis: M. R. A., E. A., A. M. B. and B. Kh.
Administrative, technical, and material support: M. R. A., E. A., A. M. B. and B. Kh.

Ethics

All procedures performed in studies involving animals were in accordance with the ethical standards of the Razi Vaccine and Serum Research Institute (Project Number: 2020-6585784-3).

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

This study was supported by Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization.

Acknowledgment

The authors would like to acknowledge and gratitude all members of the Department of Poultry Vaccines Research and Production, Razi Vaccine and Serum Research Institute, for their technical assistance.

References

1. Adams CJ, Feldman SH, Sleeman JM. Phylogenetic Analysis of Avian Poxviruses Among Free-Ranging Birds of Virginia. Avian Dis. 2005;49(4):601-5, 5.
2. Boulanger D, Smith T, Skinner MA. Morphogenesis and release of fowlpox virus. J Gen Virol. 2000;81(3):675-87.
3. Pledger A. Avian pox virus infection in a mourning dove. Can Vet J. 2005;46:1143-5.
4. Sharif A, Ahmad T. Preventing vaccine failure in poultry flocks. Immunization -Vaccine Adjuvant Delivery System and Strategies2018. p. 80-94.
5. Bolte AL, Meurer J, Kaleta EF. Avian host spectrum of avipoxviruses. Avian Pathol. 1999;28(5):415-32.
6. Manarolla G, Pisoni G, Sironi G, Rampin T. Molecular biological characterization of avian poxvirus strains isolated from different avian species. Vet Microbiol. 2010;140(1):1-8.
7. Cottingham MG, Maurik Av, Zago M, Newton AT, Anderson RJ, Howard MK, et al. Different Levels of Immunogenicity of Two Strains of Fowlpox Virus as Recombinant Vaccine Vectors Eliciting T-Cell Responses in Heterologous Prime-Boost Vaccination Strategies. 2006;13(7):747-57.
8. Skinner M, Laidlaw SM. Advances in fowl pox vaccination. CAB Rev: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources. 4. Wallingford, UK: CABI; 2009. p. 1-11.
9. Vasfi Marandi M, Bozorgmehri Fard MH. Isolation of H9N2 subtype of avian influenza viruses during an outbreak in chickens in Iran. Iran Biomed J. 2002;6:13-7.
10. Meeusen ENT, Walker J, Peters A, Pastoret P-P, Jungersen G. Current Status of Veterinary Vaccines. 2007;20(3):489-510)
11. Mockett APA, Deuter A, Southee DJ. Fowlpox. vaccination: Routes of inoculation and pathological effects. Avian Pathol. 1990;19(4):613-25.
12. Qureshi MA. Avian macrophage and immune response: an overview. Poultry Sci. 2003;82(5):691-8.
13. O'Donnell EA, Ernst DN, Hingorani R. Multiparameter Flow Cytometry: Advances in High Resolution Analysis. Immune Netw. 2013;13(2):43-54.
14. Seo SH, Webster RG. Cross-Reactive, Cell-Mediated Immunity and Protection of Chickens from Lethal H5N1 Influenza Virus Infection in Hong Kong Poultry Markets. J Virol. 2001;75(6):2516-25.
15. Skinner MA. Fowlpox Virus and Other Avipoxviruses. In: Mahy BWJ, Van Regenmortel MHV, editors. Encyclopedia of Virology (Third Edition). Oxford: Acad Pr; 2008. p. 274-84.
16. Sant AJ, McMichael A. Revealing the role of CD4+ T cells in viral immunity. Jpn J Exp Med. 2012;209(8):1391-5.
17. Dalgaard TS, Norup LR, Pedersen AR, Handberg KJ, Jørgensen PH, Juul-Madsen HR. Flow cytometric assessment of chicken T cell-mediated immune responses after Newcastle disease virus vaccination and challenge. Vaccine. 2010;28(28):4506-14.
18. Lambrecht B, Gonze M, Meulemans G, van denberg TP. Assessment of the cell-mediated immune response in chickens by detection of chicken interferon-γ in response to mitogen and recall Newcastle disease viral antigen stimulation. Avian Pathol. 2004;33(3):343-50.
19. Svahn A, Linde A, Thorstensson R, Karlén K, Andersson L, Gaines H. Development and evaluation of a flow-cytometric assay of specific cell-mediated immune response in activated whole blood for the detection of cell-mediated immunity against varicella-zoster virus. J Immunol Methods. 2003;277(1):17-25.
20. Shin JH, Park S-H. B Cells Promote Th1- Skewed NKT Cell Response by CD1d-TCR Interaction. Immune Netw. 2013;13(5):218-21.
21. Chen H-Y, Cui P, Cui B-A, Li H-P, Jiao X-Q, Zheng L-L, et al. Immune responses of chickens inoculated with a recombinant fowlpox vaccine coexpressing glycoprotein B of infectious laryngotracheitis virus and chicken IL-18. FEMS. Med Microbiol Immunol. 2011;63(2):289-95.
22. Xue M, Shi X, Zhao Y, Cui H, Hu S, Cui X, et al. Effects of Reticuloendotheliosis Virus Infection on Cytokine Production in SPF Chickens. PLoS One. 2013;8(12):e83918.
23. Kannan TA, Geetha R, Ushakumari S, Dhinakarraj G, S. V. Flow cytometric analysis of CD4+ and CD8+ T Cells in spleen of chicken (gallus domesticus). Indian J Vet Anato. 2012;24:54-5.
24. Andersen SH, Vervelde L, Sutton K, Norup LR, Wattrang E, Juul-Madsen HR, et al. Quantification and phenotypic characterisation of peripheral IFN-γ producing leucocytes in chickens vaccinated against Newcastle disease. Vet Immunol Immunopathol. 2017;193-194:18-28.