# The Effect of Trivalent Influenza Vaccines on Immunoglobulin G and Interleukin 4 in Ferret (*Mustela putorius furo*)

**Lestari Dewi[1,2], Erman Tritama[3], Reviany Vibrianita Nidom[4,5], Kuncoro Puguh Santoso[6], Sri Agus Sudjarwo[6], Harianto Notopuro[7], Chairul Anwar Nidom[4,6]**

*[1]Doctoral Student, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, [2]Lecturer, Faculty of Medicine, Universitas Hang Tuah, Surabaya, Indonesia, [3]Researcher, PT. Bio Farma (Persero), Bandung, Indonesia, [4]Researcher, Professor Nidom Foundation, Surabaya, Indonesia, [5]Researcher, PT. Riset AIRC Indonesia, Surabaya, Indonesia, [6]Lecturer, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, [7]Lecturer, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.*

## Abstract

The seasonal influenza virus has infected 5-15% of the human population every year, resulting in 250,000-500,000 deaths worldwide. The seasonal influenza epidemic that occurs every year is caused by the continuous evolution of seasonal influenza viruses, which allows them to avoid body immunity due to previous infection or vaccination, and the ability of these viruses that can be transmitted efficiently from human to human through breathing, direct contact, and through items that have been touched by sufferers of seasonal influenza. Vaccination is the most effective method for controlling the infection of seasonal influenza and the most important strategy for pandemic prevention and control. The ideal vaccine must improve the humoral and cellular immune response to reduce morbidity and mortality. This study was conducted to determine the role of trivalent influenza vaccination against the response of ferret antibodies by testing IgG levels and IL-4 levels. IgG is an antibody formed in response to vaccination, whereas IL-4 is a cytokine that stimulates B cells to differentiate into plasma cells and produce antibodies. The method of this study is an experimental laboratory. Twenty five ferrets separated into 5 group, control, trivalent vaccine dose 3.8 µg, dose 7.5 µg, dose 15 µg, and dose 30 µg. Ferrets were vaccinated with trivalent vaccine, intramuscular, 3 weeks later, that were challenge by H1N1, H3N2 and by influenza H1, H3 dan B (wild subtype) virus. On day 35$^{th}$ serum was taken and examined for IgG and IL-4. The level of IgG and IL-4 was measured by Elisa. We used SPSS 23 for data analysis. From the results of the research conducted enhancement in specific levels of IgG was obtained against H1 and H3 antigen at dose of 3.8 µg and 7.5 µg. There is no change in specific levels of IgG against By antigen. There is no change in IL-4 levels. Therefore, we conclude that IgG and IL-4 values can be used as biomarkers in testing influenza vaccines.

***Keywords:*** *immunoglobulin G, influenza vaccine, interleukin-4, Mustela putorius furo*

## Introduction

Seasonal viral influenza infections cause annual epidemics which are a significant source of public health burden throughout the world[1]. Influenza also resulted in significant economic losses. Influenza virus infections cause respiratory diseases that are highly contagious. The symptoms may be mild to severe, often leading to hospitalization and death[2]. Pneumonia is a complication that most often causes death during influenza outbreaks[3]. Influenza viruses include Orthomyxoviridae family and have genomes containing eight single stranded, negative sense RNA segments. Three types of influenza viruses A, B, and C have the ability to infect humans, although A and B are the most common circulating strains. Influenza viruses are classified based on antigenic differences in viral nucleoproteins (NP) and matrix proteins (M).

**Corresponding author:**
**Chairul Anwar Nidom**
Email: dr_lestari@yahoo.co.id; nidomca@fkh.unair.ac.id

Influenza type A viruses are further classified into subtypes by a combination of two different proteins, hemagglutinin (HA) and neuraminidase (NA), which are found on the surface of the virus. The influenza A virus subtype currently circulating among humans as seasonal influenza is influenza A (H1N1) and A (H3N2)[1,2]. Influenza B viruses can be divided into 2 main groups or lineages, B/Yamagata and B/Victoria[2].

Influenza viruses have a high evolutionary rate, and this allows the virus to mutate rapidly leading to antigenic shift and antigenic drift. The seasonal influenza epidemic that occurs every year is caused by the continuous evolution of seasonal influenza viruses, which allows them to avoid body immunity due to previous infection or vaccination, and the ability of these viruses that can be transmitted efficiently from human to human through breathing , direct contact, and through items that have been touched by sufferers of seasonal influenza[4]. The diversity of influenza virus species providing viruses has many opportunities for reassortment between subtypes, and the natural reservoir of influenza A makes elimination of disease impossible[2]. Therefore, antiviral drugs and vaccinations are more prospective solutions for controlling influenza outbreaks[5].

Vaccination is the most effective method for controlling seasonal influenza infection and the most important strategy for pandemic prevention and control[6]. Prophylactic vaccine is a tool that plays a role in preventing various infectious diseases[7]. The ideal vaccine must improve the humoral and cellular immune responses to reduce morbidity and mortality[8]. Effective vaccination in human diseases depends on the capacity of the vaccine to stimulate a protective immune response to pathogens and can produce humoral immunity and cellular immunity. In humoral immunity, B lymphocytes secrete antibodies responsible for specific recognition and neutralization pathogens derived from antigens. In addition, IL-4 is one of the most influential cytokines of the most studied immune system. The initial function of IL-4 is a B cell stimulation factor, but it is now known that these cytokines regulate a myriad of immune functions including switching immunoglobulin isotype, MHC class II expression by B cells, and the role of T cell subset differentiation from Th to Th 2[9]. In addition, ferrets and humans share similar lung physiology, and human and avian influenza viruses exhibit similar patterns of binding to sialic acids (the receptor for influenza viruses), which are distributed throughout the respiratory tract in both species. Hence, This study was conducted to determine the role of trivalent influenza vaccination against the response of ferret antibodies by testing IgG and IL-4 levels.

## Materials and Methods

Experimental Materials and Ethical Statement

This study is a laboratory experimental study with the design of pre and post test, which was conducted at Animal Biosafety Level-3, Universitas Airlangga, Surabaya, Indonesia in January 2017 - June 2017 and the Institute Medical Sciences, the University of Tokyo, Japan, in September 2017. The experimental animal treatment was carried out in Animal Biosafety Level-3, Universitas Airlangga has obtained the Animal Welfare Assurance A5966-01 for foreign institutes from the National Institutes of Health's Office Laboratory Animal Welfare (OLAW). The experimental animals used were ferret (Mustela putorius furo), female, aged 4-6 months, weight 800-900 grams. The minimum sample size used 5 ferret for each treatment group, and there were 5 treatment groups. The total sample size is 25.

**Study Design**

The experimental animals are divided into 5 treatment groups as follows:

- Group A: Trivalent vaccine dose of 3.8 µg

- Group B: trivalent vaccine dose of 7.5 µg

- Group C: trivalent vaccine dose of 15 µg

- Group D: trivalent vaccine dose of 30 µg

- Group E: without vaccine

Ferret trial animals of 25 tails were adapted for 1 week (seven days) before treatment. Day -1: Taking 3 mL of blood sample for pre-test, followed by vaccination I with trivalent vaccine, 0.5 mL with A1 dose, 3.8 µg; B1 7.5 µg; C1 15 µg; D1 30 µg; E1 with PBS by intramuscular injection in the left femur. On the 22$^{nd}$ day a challenge was tested for H1N1, H3N2, and viruses. On the 36$^{th}$ day: Euthanasia with intramuscular ketamine injection, then surgery and intracardiac blood were taken with a 10

cc syringe. Then, we were measure IgG levels with the Elisa method (using Ferrero IgG heavy and light chain Antibody, A140-108P) according to the manufacturer's instructions. The the measurement of IL-4 levels with the ELISA method (Antiferret IL-4 Ab paint No. 111132 Company; antibody research corporation) according to the manufacturer's instructions.

Processing and analysis of data

Data on IgG levels and IL-4 levels were analyzed statistically using IBM SPSS 23.

## Results and Discussion

The results of measuring levels of IgG in ferret induced by trivalent vaccine

Measuring IgG levels is done before and after vaccination. Each serum sample was tested for IgG levels 3 times, namely against H1N1 antigens, H3N2 antigens and antigens.

IgG levels against H1NI antigens before and after trivalent vaccination

IgG levels before and after trivalent vaccination with H1N1 antigens can be seen in Table 1. From the statistical test with SPSS 23, it was found that after vaccination, in testing with H1N1 antigens in groups A, B, and C there was an increase in IgG levels that were significantly different from IgG levels before vaccination. Whereas in groups D and E, there was an increase in IgG levels which were not significantly different from IgG levels before vaccination. There are differences in IgG levels between groups in measuring IgG levels with H1N1 antigens.

**Table 1. Levels of IgG against H1 antigens before and after treatment.**

| Group | n | IgG Levels on H1NI Antigens (IU/mL) | | | p (before after) |
|---|---|---|---|---|---|
| | | Before treatment | After treatment | Difference | |
| A | 5 | 2.07±0.29 | 3.03±0.06 | 0.96±0.28a | 0.002 |
| B | 5 | 2.01±0.64 | 2.98±0.09 | 0.98±0.57a | 0.018 |
| C | 5 | 2.32±0.44 | 0.07±0.05 | -2.25±0.49b | 0.000 |
| D | 5 | 2.41±0.29 | 1.37±0.91 | -1.04±1.00b | 0.081 |
| E | 5 | 2.41±0.17 | 0.18±0.15 | -2.234±0.23b | 0.000 |

Description: * significant at $\alpha = 0.05$

**IgG levels against H3N2 antigens before and after trivalent vaccination**

The results of measuring IgG levels with H3N2 antigens can be seen in Table 2. Testing with H3N2 antigens in groups A and B showed an increase in IgG levels that were significantly different from IgG levels before vaccination. Whereas in groups C, D, and E, there was an increase in IgG levels which were not significantly different from IgG levels before vaccination. There were differences in IgG levels between groups in measuring IgG levels with H3N2 antigens.

**Table 2. Levels of IgG against H3 antigens before and after treatment.**

| Group | n | IgG Levels on H3N2 Antigens (IU/mL) | | | *p* (before after) |
|---|---|---|---|---|---|
| | | Before treatment | After treatment | Difference | |
| A | 5 | 1.66±0.27 | 2.96±0.13 | 1.30±0.22[a] | 0.000 |
| B | 5 | 1.52±0.49 | 2.91±0.14 | 1.40±0.42[a] | 0.002 |
| C | 5 | 1.90±0.22 | 0.06±0.01 | -1.84±0.23[c] | 0.000 |
| D | 5 | 1.93±0.40 | 0.95±0.98 | -0.984±0.97[c] | 0.080 |
| E | 5 | 2.11±0.14 | 0.11±0.07 | -1.99±0.16[b] | 0.000 |

Description: * significant at α = 0.05

**Level of IgG against B antigen (yamagata) before and after trivalent vaccination**

The results of measuring IgG levels with antigen By can be seen in Table 3 below. Testing with antigen By in group B there was an increase in IgG levels that were significantly different from IgG levels before vaccination. Whereas in groups A, C, D, and E, there was an increase in IgG levels which did not differ significantly from IgG levels before vaccination. There was no difference in IgG levels between groups in measuring IgG levels with H3N2 antigens.

Table 3. Levels of IgG against antigen By before and after treatment.

| Group | n | IgG Levels on ByAntigens (IU/mL) | | | *p* (before after) |
|---|---|---|---|---|---|
| | | Before treatment | After treatment | Difference | |
| A | 5 | 1.73±0.47 | 0.98±0.54 | -0.75±0.81[a] | 0.108 |
| B | 5 | 1.64±0.42 | 2.01±0.10 | 0.37±0.44[a] | 0.131 |
| C | 5 | 1.45±0.28 | 1.83±0.24 | 0.38±0.45[a] | 0.133 |
| D | 5 | 1.66±0.34 | 1.96±0.21 | 0.30±0.37[a] | 0.141 |
| E | 5 | 1.79±0.17 | 1.75±0.17 | -0.04±0.13[a] | 0.508 |

Description: * significant at α = 0.05

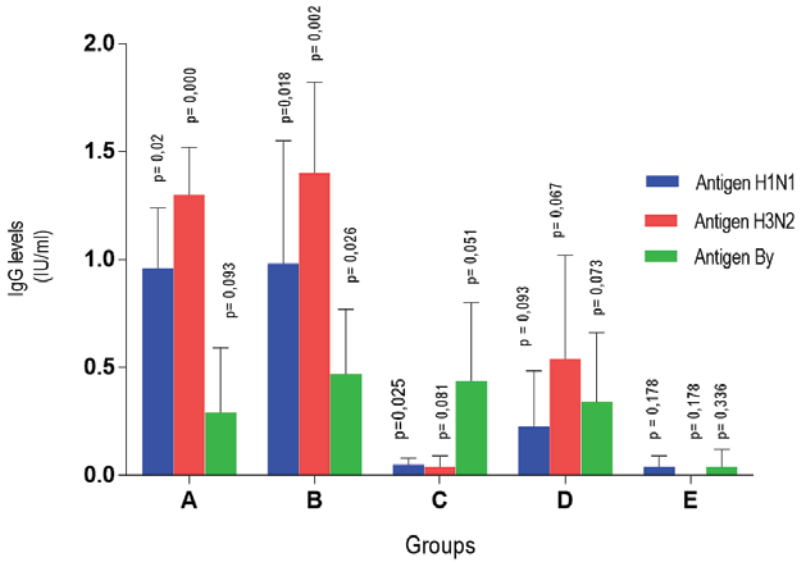


**Figure 1. IgG levels of each group against H1N1, H3N2 and By.**

Based on Figure 1 above, trivalent vaccines of 7.5 µg (group B) and 3.8 µg (group A) doses can induce high levels of IgG in all three antigens.

**IL-4 levels after trivalent vaccination in ferret**

The results of measurement of IL-4 levels can be seen in table 4 below. IL-4 levels in groups A, B, C, and D showed a significantly different increase than before vaccination. In group E, there was no increase in IL 4 levels which was not significantly different from before vaccination. There were differences in IL4 levels between groups after trivalent vaccination.

**Table 4. Levels of IL-4 before and after trivalent vaccination.**

| Groups | n | IL-4 (pg/mL) | | | |
|---|---|---|---|---|---|
| | | Before treatment | After treatment | Difference | p (before after) |
| A1 | 5 | 0.047±0.003 | 0.048±0.007 | 0.0016±0.006a | 0.304 |
| B1 | 5 | 0.050±0.005 | 0.063±0.042 | 0.013±0.040a | 0.515 |
| C1 | 5 | 0.084±0.083 | 0.095±0.081 | 0.011±0.010a | 0.08 |
| D1 | 5 | 0.046±0.008 | 0.052±0.005 | 0.005±0.010a | 0.329 |
| E1 | 5 | 0.049±0.007 | 0.050±0.006 | 0.001±0.010a | 0.871 |

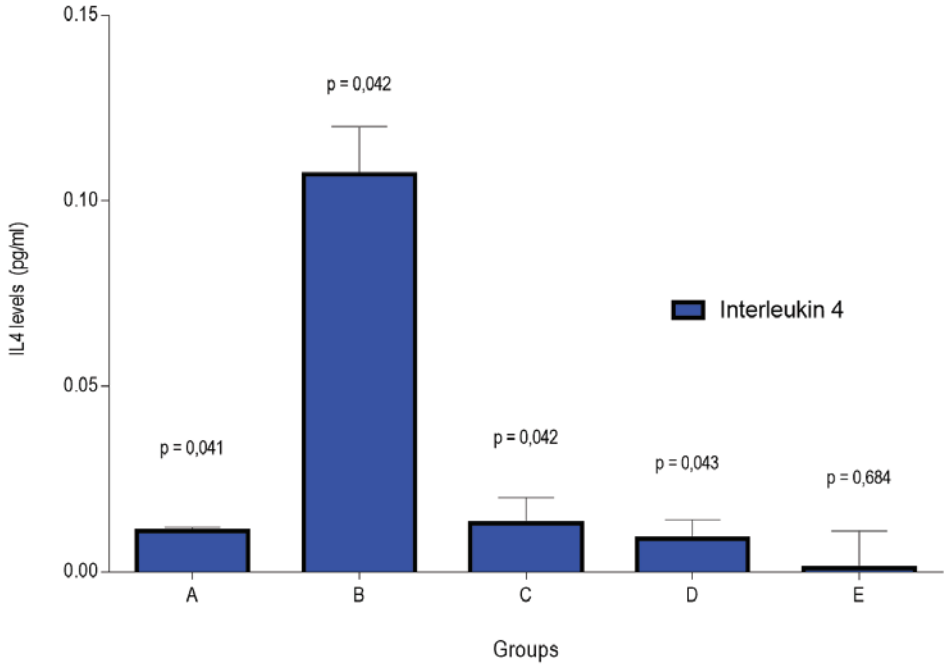


**Figure 2. Increased levels of IL4 from each group after trivalent vaccination.**

From Figure 2, the experimental group that received a dose of 7.5 µg, had the highest increase in IL4 levels after trivalent vaccination. Based on the results of research that has been done, before trivalent vaccination, the serum already contains IgG antibody titers. The response was due to differences in pre-vaccination memory deposits among subjects[10]. In measuring IgG levels with all three antigens, group B who received vaccinations at a dose of 7.5 µg showed being able to induce ferret to produce the highest levels of IgG from the five groups, and the IgG levels differed significantly from IgG levels before vaccination. There is an increase in IgG levels in all groups, and in all antigens, but not all are significantly different.

In group E, who did not get vaccinations, but received a challenge test with trivalent influenza virus, showed a slight increase in IgG levels, and did not differ significantly from IgG levels before treatment. The main function of B lymphocytes is producing antibodies. Another name for antibodies is immunoglobulin, which is a glycosylated protein molecule found on the surface of B cells, functions as an antigen receptor, or secreted also to the extracellular space so that it can bind and neutralize the antigen. A single antibody molecule consists of four protein chains, 2 heav chain, 2 light chains. Antibodies consist of 5 isotypes or classes, namely IgM, IgD, IgA, IG and IgE[11]. Anti-influenza antibody titers and cross reactivity with new viruses are routinely used to assess the potential for protection against new influenza virus infections. When combined with the characteristics of viral genome diversity, serological tests can provide insight into immunity stimulated by the evolution of the virus.

The results of measurements of IL-4 levels showed that there were differences in IL 4 levels between groups after trivalent influenza vaccination. The dose groups 3.8 µg, 7.5 µg, 15 µg, and 30 µg had increased IL4 levels after vaccination and differed significantly compared to group E who did not get vaccinations. Group B with a dose of 7.5 µg had the highest IL4 level. Based on the type of cytokines produced, Thelper lymphocytes are divided into Th1 which produces proinflammatory cytokines TNF-α and TNF-β which are now called lymphotoxins, IFN-γ, IL-1, IL-6, IL-8, IL-12, which function to activate cellular immunity and non specific. Besides that there is Th2 which produces anti-inflammatory cytokines, namely IL-4 and IL-10 which activate humoral immunity[12].

IL-4 is one of the most influential cytokines of the most studied immune system. Initially as a B cell stimulation factor, it is now known that this cytokine

regulates a myriad of immune functions including switching Ig isotype, class II MHC expression by B cells, and the role of T cell subset differentiation from Th to Th 2. The presence of IL-4 in the early stages of the immune response has an important effect on the natural immune response. IL-4 supports the further production of IL-4 by T cells, stimulates antibody formation, and it inhibits IFN-γ production, thus inhibiting hypersensitivity reactions. IL-4 is a cytokine released by various types of cells including T cells, eosinophils, basophils, mast cells, natural killer cells (NK) and several antigen presenting cells (APC)[6].

## Conclusion

In summary, we conclude that IgG and IL-4 values can be used as biomarkers in testing influenza vaccines.

**Conflict of Interest:** The authors declare that they have no conflict of interest.

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**Ethical Approval**: The experimental animal treatment was carried out in Animal Biosafety Level-3, Universitas Airlangga has obtained the Animal Welfare Assurance A5966-01 for foreign institutes from the National Institutes of Health's Office Laboratory Animal Welfare (OLAW).

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