Journal Homepage: - www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI: 10.21474/IJAR01/16458
DOI URL: http://dx.doi.org/10.21474/IJAR01/16458

RESEARCH ARTICLE

EFFECTOF EHV VACCINE ON IMMUNOLOGICAL PARAMETERS IN ARABIAN FOALS

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Manuscript Info

Manuscript History
Received: 19 January 2023
Final Accepted: 24 February 2023
Published: March 2023

Key words:-
Arabian Horse, Vaccine, Immunoglobin, Toll Like Receptor, Interleukin

Abstract

By mimicking the infections, vaccines can offer protection from some diseases. This sort of imitating infections helps to teach the immune system how to combat an infection in the future.Discovery of immune responses linked to vaccination protection in Arabian horses has been a long-standing objective for scientists**.**The purpose of this study was to investigate the changes of immunological parameters before and after vaccination by **Fluvac Innovator®** vaccine in Arabian foals. Twenty healthy, Arabian foals of both sexes were examined (before vaccination and after vaccination) 20 blood samples were examined before vaccination and 20 blood samples were examined at 45 days of vaccination. Leukogramwas performed. Immunoglobulins including Ig E, Ig G, Ig A and Ig M were measured in serum and genes expression using Real Time-PCR were performed for IL-10, IL-6, TNFα and TLR 4 mRNA.After vaccination, there were significant increases in lymphocytes, IgG, IgM, IL-10, IL-6, TNF-α, TLR 4 mRNA expression in males and females when compared to basal value before vaccination. therefore, assessing humoral response (immunoglobins A, M, G and E) and cellular response (WBCs, IL-10, IL-6, TNF- α and TLR-4) is an important tool for determination **Fluvac Innovator®** vaccine effect on immunity.

Introduction:-

Infectious diseases have been able to travel across international borders thanks to the transboundary trafficking of arabian horses from various regions of the world during horse events like races and displays**(Oladunni et al., 2021).**

Equine influenza (EI), which is regarded as a major cause of respiratory infection in horses worldwide, sprang from these viruses. EI is exceedingly contagious and can result in up to 100% morbidity, but it can be fatal to young animals and older horses with impaired immune systems. Continuous EI outbreaks have recently been recorded in a number of nations, including the USA, Europe, Asia, and African nations**(Paillot et al., 2019).** Also, Equine herpesvirus (EHV) is an alpha herpesvirus that infects horses and causes neurological and respiratory illness as well as abortion in mares that are pregnant**(Khusro et al., 2020).**

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Horse veterinarians are confronted by new commercial vaccines, new efficacy claims, potential adverse effects, as well as an improved awareness of the dubious efficacy of old vaccines. The immune system is boosted by vaccinations by fooling the body into thinking that an infection is present, and an efficient vaccine should stop disease and viral shedding from the animal by activating the immune system as well. such that defense systems like antibodies and other immune mechanisms are activated, while booster shots afterward are anticipated to activate a memory immune response **(Davis et al., 2015).**

For vaccination to be effective, both humoral and cellular immune responses are required**(Khusro et al., 2020).**The detection of immunoglobins, which are glycoproteins made by plasma cells after being stimulated by an antigen, is a component of the humoral immune response. They are additionally known as antibodies. IgG, IgM, IgA, Ig D, and Ig E are the five main Ig classes that have been identified in horses **(Horohov, 2015).**While cellular immune response includes leukocytes subtypes**(Giannetto et al., 2022)**and cytokines including IL-10,IL-6 and TNF α **(Migdał et al., 2020).**Also**,**a family of innate immune system receptors with germline coding is known as toll-like receptors (TLR).**(Jungi et al., 2011)**

The FLUVAC INNOVATOR inactivated vaccine broadens the protection offered to arabian horses against both novel and prevailing strains of equine influenza virus (EIV) as well as equine herpesvirus (EHV-1 and EHV-4)**(Selvaraj, 2019).**

Therefore, this study's objective was to investigate the immunological response in arabianhorses after vaccination with FLUVAC INNOVATOR through assessing humoral response (immunoglobins A, M, G and E) and cellular response (WBCs, IL-10, IL-6, TNF- α and TLR-4)

Materials and Methods

Ethical Approval/Animal Welfare:

The investigation was conducted in accordance with the biomedical research guidelines for the care, and the Clinical Pathology Department of the College of Veterinary Medicine at Benha University's Ethics Committee in Animal Experiments accepted the experimental methodology. (No. BUFVTM 21-10-22).

The usual vaccination and blood sampling of horses during this study, which was conducted with the farm's supervising veterinarian's permission, remained unchanged. In the equine stable, immunization and sample collection were done. During the trial, horses were kept in their stables, fed regularly during the off-racing season, and given access to unlimited amounts of water. Following vaccination and sample collection, horses were closely watched for the emergence of any unfavorable disease symptoms.

Experimental design:

In this research, twenty healthy,male and female arabian foals. Aging between 3-9 months and the weight of between 80-150 kg were numbered and looked at as follows::

20 foals (10males and 10 females) did not receive any types of vaccine and blood samples examined before vaccination. The same animals received a 1cm dose of the inactivated Fluvac Innovator® vaccine (Zoetis LLC, Parsippany, NJ, USA), which contains A/equi-2/Kentucky/97, EHV-1, and EHV-4and MetaStim® was used as an adjuvanttaken intramuscular injection. Also, samples were taken after 45 day of vaccination **(Pavulraj et al., 2021).**

Blood sampling:

From the jugular vein, 40 blood samples were aspiratedwith a one inch, 18to20gauge needle (20 samples before vaccination and 20 samples at 45 day of vaccination) For determination complete blood count (CBC)about 5 ml bloods was taken using vacuum tubes (Vacutainer®) which have 3.6 mg dipotassium EDTA and applied to determination of leukogram **(Draeger, 2020)**.Moreover, 5 ml of blood were drawn into a plain, clean, well-dried centrifuge tube to separate the serum for estimation of the biochemical parameters. After allowing the blood samples to coagulate, serum samples were extracted using a centrifuge at 3000 revolutions per minute for 15 minutesSera were acquired using an automated pipette, put into Eppendorf tubes that were clean, dry, and labelled, and then preserved in the freezer until evaluation of immunoglobulins: Ig M , Ig G **(Buening et al., 1977)** , Ig E **(Hoffman, 1981)** , Ig A **(Staley et al., 2018)**.

PCR:

RNA extraction:

The QIAamp RNA-blood extraction kit was used to extract RNA from whole blood (Qiagen, Germany, GmbH). The procedures were carried out in accordance with the QIAamp RNA-blood extraction kit's Purification of Total RNA protocol. Primers used were supplied from **Invitrogen by Thermo Fisher Scientific** are listed in table **(Vendrig et al., 2013).**

Target gene	Sequence
B- actin	F 5′-CAAGGCCAACCGCGAGAAGATGAC-3′
	R5′-GCCAGAGGCGTACAGGGACAGCA-3′
TNF-α	F 5′-TCCAGACGGTGCTTGTGC-3′
	R5′-GGCCAGAGGGTTGATTGACT-3′.
IL6	F 5′-TGGCTGAAGAACACAACAACT-3′
	R 5′-GAATGCCCATGAACTACAACA-3′.
IL10	F 5′-GAGAACCACGGCCCAGACATCAAG-3′
	R 5′-GACAGCGCCGCAGCCTCACT-3′.
TLR 4	F 5′-CCCTTTCAACTCTGCCTTCACT-3′
	R 5′-GGGACACCACGACAATAACTTTC-3′.

SYBR green rt-PCR

20 µl reaction consisting of 10 µl of the 2x HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 0.5 µl of each primer of 20 pmol concentration,1 µl of RT Enzyme Mix (20X), 0.5 µl dye, 2.5 µl of water, and 5 µl of RNA template. The reaction was carried out in a step-one real time PCR machine.

Conditions for SYBR green rt-PCR:

Reverse transcription was done at 45˚C/ 5 min. Also, Primary denaturation at 94˚C/ 30 sec meanwhile, Amplification (40 cycles) consists of denaturation at 94˚C/ 5 sec, Annealing at 55˚C/ 15 sec and Extension at 72˚C/ 10 sec.

Statistical analysis:

The statistical evaluation was completedusing T-testthrough SPSS, ver. 25 IBM Corp. Released 2013Data were handled as a complete randomization design according to **(Steel and Torrie, 1980))**.The significance level was set at < 0.05.

Result:-

Blood samples were collected before vaccination (basal value) and after 45 days from vaccination of **Fluvac Innovator®** vaccine (ZOETIS) in males and females.

The results that were attained are as follows:

Influence of the vaccination on the leukogram:

Data showing the influence of vaccine on leukogramare listed in table(1)

Vaccinated Males demonstrated significant increase in lymphocytes and non-significant increase in WBCs count, absolute number of monocytes, eosinophils, and granulocytes. While vaccinated females showed very high significant increase in lymphocytes, high significant increase in eosinophils and no significant increase in wbcs count and monocytes. Also showed high significant decrease in granulocytes when compared to their corresponding pre-vaccinevalue of males and females respectively.

Influence of the vaccination on immunoglobulins:

Data showing the influence of vaccine on immunoglobulins are listed in table (2)

Vaccinated Males showed significant increase in Ig E and Ig M, very high significant increase in Ig G and no significant increase in Ig A when compared with their corresponding basal value. Meanwhile,vaccinated females showed significant decrease in Ig A and very high significant decrease in Ig E. Also, showed high significant increase in Ig G and very high significant increase in Ig M when compared to their corresponding pre-vaccine value.

Influence of the vaccination on expression of m RNA:
Data showing the influence of vaccine on expression of m RNAare listed in table (**4**)

Vaccinated Males showed very high significant increase in TNF-α by 12.87 folds, significant increase in IL-6 by 16.08 folds, significant increase in IL-10 by 3.09 folds and high significant increase in TLR-4 by 2.76 folds when compared to their corresponding basal value.

According tovaccinated females, they showed high significant increase in TNF-α by 1.82 folds, high significant increase in IL-6 by 3.93 folds, significant increase in IL-10 by 1.94 folds and high significant increase in TLR-4 by 1.25 folds when compared to their corresponding basal value.

Discussion:-

In many nations, particularly in Egypt and other Middle Eastern nations, the equine sector is significant commercially. EHV-1 results in respiratory problems, mares aborting, weak, nonviable foals being born, and even sporadic paralytic neurologic illness **(Mesquita et al., 2017).** The greatest method for preventing and controlling disease outbreaks is effective immunization combined with competent animal management, particularly when utilizing the inactivated vaccine in endemic areas **(Nehal, 2006).**

When the body is exposed to antigens through vaccination or other means, macrophages and dendritic cells are stimulated, which results in the production of inflammatory cytokines and the induction of APP synthesis in hepatocytes, which serve as a generalized component of the innate immune system that can detect pathogens or vaccination components**(Alsemgeest et al., 1994).**There is evidence that vaccines trigger inflammatory reactions, which directly affect the maintenance of homeostasis, especially in the liver and kidney **(Mills et al., 1998).**

According to recommended practices, the majority of the horses in this study received their first vaccination shot between the ages of 3 and 9 months. Young seronegative horses are more vulnerable to infection and may have a significant impact on virus dissemination, hence it is advised that vaccination against EI begin once maternally derived antibodies (MDA) have diminished**(Gildea et al., 2013).**

The results of the present investigation demonstrated a dynamic alteration in leukocyte populations, including lymphocytes, neutrophils, and eosinophils, following vaccination.

In the current research, lymphocytes demonstrated significant increase in vaccinated males ($p<0.05$) and very high significant increase in vaccinated females ($p<0.001$) when compared with their corresponding basal valueofmales and females respectively.Our findings concur with **Goundasheva et al., (2005)**and **Giannetto et al., (2022)**who claimed that a lymphocytes showed significant increase compared to basal values was observed on 28th days after vaccination.

It is not surprising that these leukocyte subtypes increased after immunization, especially given the functions they carry out. The proliferation of blood lymphocytes induced by viral proteins known to behave as mitogens or superantigens on some lymphocyte subpopulations was likely a factor in the immunological activation following vaccination **(Cook, 2008).**

In according to neutrophils,they showed high significant decreasein vaccinated females ($p<0.01$) in comparison to their corresponding basal value of non-vaccinated females but there is no significant change in vaccinated males.The decrease of neutrophils in vaccinated females agrees with **Giannetto et al., (2022)**who reported that neutrophils showed significant decrease compared to basal values.

From a clinical perspective, neutrophils have certain essential characteristics in common. Patients are shielded by these cells from an enormous danger of deadly infections. Before being delivered into the phagosome, digestive and hydrolytic enzymes are stored in neutrophil granules **(Weiss and Wardrop, 2011)**.The body's immuno-biologic transformation, which is consistent with the significant increase in the lymphocyte percentage, can be used to explain these alterations**(Goundasheva et al., 2002).**

According to eosinophils, they showed high significant increase in vaccinated females (p<**0**.01) when compared with their corresponding basal value of females. But in vaccinated males , there is no significant increase when compared totheir corresponding basal value.

The increase of eosinophils in females agrees with **Burakova et al., (2018)**who reported that eosinophils increased after vaccination by inactivated vaccine. This is attributed to the vaccine may cause allergic reaction which cause eosinophilia **(Fettelschoss-Gabriel et al., 2018).**Although their effectiveness against intracellular pathogens is quite limited, there are numerous reports on the use of aluminium adjuvants in vaccines against economically significant viral illnesses, such as Newcastle disease and foot-and-mouth disease (FMD)**(Lindblad, 2004)**.Interleukin (IL)-4, a cytokine that enhances the T helper 2 (Th2) immunological response, is secreted more readily when aluminium adjuvants are present. Eosinophils and IgG and IgE immunoglobulins are produced as a result **(Ulanova et al., 2001).**This makes these adjuvants suitable choices for vaccinations against germs and parasites. **(Gupta, 1998).**

It has been demonstrated that serum antibody measurement is highly sensitive for identifying influenza infection and useful for assessing the value of vaccine-induced protection**(Newton et al., 2000).**

The creation of immunoglobulins like Ig M, Ig G, and Ig A is linked to an increase in the -globulin fraction associated with lymphocytosis, which allows one to infer that vaccination increases B lymphocytes, which in turn stimulates the production of antibodies**(Eckersall, 2008) and (Giannetto et al., 2022)**

In this investigation, the levels of Ig E of vaccinated males showed significant increase (p<**0**.05) but showed very high significant decrease (p<**0**.001) in vaccinated females when compared to their corresponding basal value.

The increase of concentrations of Ig E in vaccinated males agrees with **(Vogel and Powell, 1995))** who reported thatthere is increase in Ig E in males group after vaccination when compared to basel value**.**This may be attributable to adjuvants, which promote the generation of IL-4 and activate Th2 cells with higher levels of IgG and IgE.

In this study, the concentrations of Ig E is the lowest value compared with other immunoglobins in the serum. This result agrees with **Wagner et al., (2003) and Wagner et al., (2006)**who reported that Ig E is one of the minor parasite immunity factors in horses and is present in serum in the smallest concentration. It participates in pathological reactions such as IgE-mediated hypersensitivities in horses and is thought to prevent parasite infection**(Hellberg et al., 2006).**

In this investigation, the levels of Ig A of vaccinated males showed non-significantincreasebut showed significant decrease (p<0.05) in vaccinated females in comparison with their corresponding basal value.

Horse mucosa, saliva, tears, and sweat are the main sources of IgA. On mucosal surfaces, macrophages that have the ability to opsonize antigens can then phagocytize those antigens. Because IgA and IgE interact so intimately, if IgA synthesis is low, an IgE response may cause the formation of excessive levels of IgE.Low levels of IgA cause increased Ig E production as a result of this delicate balance, which can also cause allergic reactions to develop to foods or inhaled antigens**(Cunha et al., 2006).**

Regarding to Ig A in males group, our findings concur with **Muirhead et al., (2008)**who claimed that There were no notable changesin anti influenza IgA titers after vaccination .The biological significance of this observation is unclear because the serum IgA titers were much lower than the IgG titer in actuality. Ig A immunoglobulin is primarily present on mucosal surfaces, where it is most effective at preventing respiratory viral infections **(Janeway et al., 2001).**

In this investigation, the levels of Ig G of vaccinated males showed very high significant increase (p<0.001) also, showed high significant increase (p<0.01) in vaccinated females when compared to their corresponding basal value.

Interestingly, the concentrations of Ig G is the highest value compared with other immunoglobins in the serum. Our result concur with **Sheoran et al., (2000)**by whom was that saidIn equine serum and colostrum, IgG predominates. It is also present on mucosal surfaces, including those of the urinary system, lower respiratory tract, and lung.The predominant immunoglobulin of horses in serum is Ig G. Equine herpesvirus type 1 (EHV-1) and EHV-4, as well as the equine influenza virus, are among the many horse infections for which Ig G is essential in neutralising immune

responses**(Goehring et al., 2010)**, Rhodococcusequi , Theileriaequi**(Mealey et al., 2012)** and nematodes **(Dowdall et al., 2002).**

Also Our findings are consistent with **Su et al., (2008)**by whom was that saidafter vaccination there was high concentration of Ig G.The identification of EHV-1 neutralising antibodies in serum samples from groups of vaccinated horses within 6 months post- vaccination, rise ≥ 4 fold titer rise when compared with their corresponding non vaccinated horses to provide a correlate of protection against EI infection**(Abousenna et al., 2022)**.

In this investigation, the levels of Ig M of vaccinated males showed significant increase ($p<0.05$) and showed very high significant increase ($p<0.001$) in vaccinated females when compared to their corresponding basal value.

Ig M is increased after vaccine in both groups ,Our result concur with**Giannetto et al., (2022)**by whom was that saidimmunoglobulins production such as IgM occur after vaccination by inactivated vaccine .The principal immunoglobulin produced during an initial immune response is IgM. Moreover, it is created in a supplementary response. IgM is the first isotype to be expressed both during the formation of B-cells and the initial immunological response to an infection. The mucosal immune response's main antibody is IgM **(Fermaglich, 2003).**

The majority of horse cytokines cannot yet be measured using commercially available enzyme-linked immunosorbent assays (ELISA). Real-time qRT-PCR is now widely used to measure mRNA levels in gene expression investigation**(Quinlivan et al., 2007).**

Vaccinations are used to reduce the severity and duration of future disease in humans and animals by presenting a weakened or killed version of a virus to the immune system. Previous studies show that the immune response can be affected by the concentration of chemical messengers, called cytokines in the blood **(Goodman et al., 2012).**

In this study, there is very high significant expression of TNF-α mRNA in vaccinated males ($p<0.001$) and high significant expressionin vaccinated females ($p<0.01$). Also, corresponding to IL-6,there is significant expression of TNF-α mRNA in vaccinated males ($p<0.05$) and high significant expressionin vaccinated females ($p<0.01$) when compared with their corresponding basal value of males and females respectively.

Our result concur with**Su et al., (2008)**by whom was that saidafter vaccination there was highly expression for TNF-α and IL-6.Also, For boosting innate immunity against viral infections, TNF- and IL-6 are ideal choices. **(Trevejo et al., 2001).**The cytokines IL6 and TNF- function as co-stimulatory agents that increase IgG production as well as humoral and cellular responses and this confirm our result for increasing concentration of Ig G after vaccination. Both cytokines increase T cell proliferation at greater levels and cytotoxic responses at higher levels. also increased the production of IFN-γ in CD4+ and CD8+ T cell subsets **(Su et al., 2008).**

According to IL-10, there is significant expression of IL-10mRNA in vaccinated males and vaccinated females ($p<0.05$) when compared with their corresponding basal value of males and females respectively.

Our result concur with**Brummer et al., (2013) , Wagner et al., (2017)and Stefansdottir et al., (2022)**by whom was that saidIL-10 and IL-6 were highly expressed after vaccination with an influenza virus strain.Also,Upon immunization, there was a noticeable rise in cytokine responses, including the release of IL-10 and IFN-γ. Adjuvant tended to up-regulate the genes encoding IL-1β,TNF- α, IL-6, IL-8 and IL-10 in equine as it does in human and murine cells (**(Hellman et al., 2018)).**One of the most significant anti-inflammatory cytokines is the cytokine IL-10**(Opal and DePalo, 2000)**.

Regarding to TLR-4,there is high significant expression of TLR-4 mRNA in vaccinated males ($p<0.01$) and also significant expressionin vaccinated females ($p<0.05$) when compared with their corresponding basal value of males and females respectively.

Our result concur with**Reed et al., (2009)**by whom was that saidadjuvant can stimulate TLR-4 expression Th2 and B-cell responses are more likely to be induced by adjuvants, Conversely, TLR ligand-containing PAMPs support higher Th1 and immunological responses.

Pathogen-recognition receptors (PRRs), which include the Toll-like receptors, let the immune system identify pathogen-associated molecular patterns (PAMPs) (TLRs)**(Kawai and Akira, 2007)**, C-type lectin-like receptors **(McGreal et al., 2005)**,cytosolic nucleotide oligomerization domain-like receptors and retinoic acid inducible genebased-I-like receptor**(Carneiro et al., 2007).** These receptors bind microbial ligands (including cell wall components, lipoproteins, proteins, lipopolysaccharides, DNA and RNA of bacteria, viruses, protozoa and fungi) to trigger different types of immune responses **(Pålsson-McDermott and O'Neill, 2007).**The foundation of many adjuvants is these PAMPs, particularly those that bind to TLRs.Moreover, cytokines, bacterial toxins, and glycolipids that change the way an antigen is processed are employed in adjuvants to trigger an immune response**(Ishii and Akira, 2007).**Ten TLRs (TLR-1 through TLR-10) have been identified as activating various signalling pathways that have various biological consequences. The most researched TLR, TLR-4, can detect the following substances in addition to others: LPS1 from bacteria, lipid A, and its less harmful chemically modified derivatives**(Kawai and Akira, 2004).**

An immune response is triggered by the recognition of the threat and the activation of TLRs, which results in the removal of the threat from the organism. This immune response consists of two main reactions, inflammatory and antiviral.The proinflammatory cytokines IL-1, -6, -8, -12, and TNF-α are produced in greater amounts when the TLRs on the surface of macrophages are activated**(Migdał et al., 2020).**Interestingly,the production of IL-6 was dependent on viral recognition by Toll-like receptors **(Farsakoglu et al., 2019)**.This confirm our result for increasing expression of both IL-6 and TLR-4.

Conclusion:-

The FLUVAC INNOVATOR inactivated vaccine provides the arabian horses with strong immunity either cellular immunity which includes increase in lymphocytes and highly expression of TNF-α,IL-6,IL-10 and TLR 4 mRNA . Also stimulate humoral immunity which includes increasing concentration of IgG and IgM.

Also, there is a difference in immune response in males and females arabian horses after vaccination by FLUVAC INNOVATOR either in wbcs ,immunoglobulins levels and expression of TNF-α,IL-6,IL-10 and TLR 4 mRNA

Table (1):- Leukogram before and after vaccination in male and female Arabian foals:

Parameter	Before vaccine	After vaccine
Males		
WBCs ($x10^3$/µl)	11.90±0.53	12.70±0.53
LYM ($x10^3$/µl)	7.11±0.56	8.74±0.42*
Mon ($x10^3$/µl)	0.25±0.07	0.21±0.03
EO ($x10^3$/µl)	0.41±0.08	0.60±0.07
GR ($x10^3$/µl)	4.21±0.53	3.14±0.44
Females		
WBCs ($x10^3$/µl)	12.02±0.45	12.75±0.46
LYM ($x10^3$/µl)	6.5±0.44	9.04±0.38***
Mon ($x10^3$/µl)	0.24±0.06	0.33±0.05
EO ($x10^3$/µl)	0.26±0.07	0.55±0.06**
GR ($x10^3$/µl)	5.03±0.6	2.84±0.35**

*: Significant **: High significant ***: Very high significant

Table (2):- Igsbefore and after vaccination in male and female Arabian foals:

Parameter	Before vaccine	After vaccine
Males		
IgE (g/l)	0.14±0.03	0.26±0.03*
IgA (g/l)	5.37±0.23	5.17±0.22
IgG (g/l)	464.08±8.48	567.21±6.75***
IgM (g/l)	61.41±7.77	87.58±6.67*
Females		
IgE (g/l)	0.10±0.02	0.02±0.00***
IgA (g/l)	5.24±0.18	4.71±0.14*
IgG (g/l)	464.12±6.15	529.18±10.01**

IgM (g/l)	60.19±7.97	87.89±1.36***

*: Significant **: High significant ***: Very high significant

Table (3):- Genes expression before and after vaccination in male and female Arabian foals by fold change:

Parameter	Before vaccine	After vaccine
Males		
TNF-α	1.00±0.00	12.87±0.85***
IL6	1.00±0.00	16.08±5.12*
IL10	1.00±0.00	3.09±0.51*
TLR4	1.00±0.00	2.76±0.25**
Females		
TNF-α	1.00±0.00	1.82±0.10**
IL6	1.00±0.00	3.93±0.62**
IL10	1.00±0.00	1.94±0.30*
TLR4	1.00±0.00	1.25±0.04**

*: Significant **: High significant ***: Very high significant

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