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PRODUCTION OF FOOT-AND-MOUTH DISEASE (SEROTYPE: O, A AND SAT-2) POLYCLONAL ANTIBODIES (IgY) BY USING LAYER HENS

MSc Thesis

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

BY

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SEPTEMBER, 2020

BISHOFTU, ETHIPIA

PRODUCTION OF FOOT-AND-MOUTH DISEASE (SEROTYPE: O, A AND SAT-2) POLYCLONAL ANTIBODIES (IgY) BY USING LAYER HENS

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

By:

Ephrem Shimelis Abebaw

SEPTEMBER, 2020

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Signature and Approval Sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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Title: **Production of Foot-and-mouth disease (Serotype: O, A and SAT-2) polyclonal antibodies (IgY) by using layer hens**

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LIST OF ABBREVIATION

BALT: bronchial associated lymphoid tissues

CPE: Cytopathic effect

Cre: cis-acting replication element

DC: Dendritic Cell

DIVA: Differentiation of infected animals from vaccinated animals

FMDV; Foot-and-mouth disease virus

GALT: Gut associated lymphoid tissues

IRES: Internal ribosome entry site

ISG: interferon-stimulated genes

Mab: Monoclonal antibody

MALT: mucosal surfaces associated lymphoid tissues

nFPC: net OD of the positive control

ORF: an open reading fragment

PAb: Polyclonal antibody

PFU: Plaque forming Unit

PI: Percentage of Inhibition

SVD: swine vesicular disease

TCID: tissue culture infectious dose

UTR: untranslated region

VNT: Virus neutralization tests

VS: vesicular stomatitis

ABSTRACT

An experimental study on FMDV (O, A and SAT-2) polyclonal antibody production trials was conducted from January, 2020 up to August, 2020 G.C. The objective of this study was production of polyclonal antibody from chickens immunized with FMDV. The experimental chickens were grouped in to four where each group consists of eight birds except the control group an inoculated at day 0 and day 14 with quantified dose of serotype O; A, and SAT-2. The immunized chickens were sampled at day 14, 21 and 28 post immunization. Totally 144 sample (72 serum and 72 egg yolk) were pooled by two according to its collection day, serotype and antibody sources in order to make laboratory tests feasible. Non-structural protein detection using ELISA showed the percentage of inhibition (PI) less (< 50%) for all samples detected. At day 14, 21 and 28 post immunization the mean Egg yolk IgY were 10, 8.6 and 13.46 respectively. The result of VNT titer indicated that samples with less Percentage of inhibition, neutralized the homologous FMDV serotypes (Titer TCID log10 > 0.3). On the other hand, the mean antibody titer at day 14, 21 and 28 were 1.83, 2.1 and 2.07 respectively. Even though antibody percentage of inhibition and antibody titer were low, there was a linear relationship between the egg yolk IgY and serum antibody titer. The difference between egg yolk IgY and serum antibody were only significant as compared by the FMDV serotypes (P-Value < 0.05). The study result indicated that IgY antibody could be used for any immunological tests applied for FMD diagnosis. Moreover, the study finding indicated that chicken egg yolk antibody (IgY) production could be the best alternative to the other mammalian laboratory animals as egg yolk-based antibody production reduce the stress and bleeding during the blood collection, high antibody titer and low cost, easy to manage and handle chickens.

Key words: *FMDV serotype A, O and SAT-2, antibody, egg yolk IgY, serum, Percentage of inhibition and antibody titer.*

1 INTRODUCTION

The agro-climatic conditions of Ethiopia make it very suitable for the production of different kinds of livestock and to have the largest livestock population in Africa (CSA. 2017). The estimated livestock population data indicate that 53.99 million heads of cattle, 25.49 million heads of sheep, 24.06 million heads of goats, 50.38 million heads of poultry,1million heads of camel and 5.21 million beehives in Ethiopia (Asegede *et al*.,2015). Livestock the economic, social, and cultural lives of millions of poor farmers depend on livestock (Addis *et al*., 2017). The contribution of the livestock and its production to the national economy is minimal compared to its potential. The main constraint for this is the widespread occurrence of many infectious diseases, such as foot and mouth disease (FMD), which drastically reduces the production and productivity of livestock (Tadesse *et al.,* 2019).

The foot-and-mouth disease virus (FMDV) is belonging under the family of Picornaviridae and genus of *Aphtho* virus (Aftosa, 2015) also there are seven main serotypes of FMDV (A, O, C, Asia1, South African Territories (SAT) 1, SAT2, and SAT3),which resulted from the high mutation rate of FMDV with its rapid proliferation and extensive population(Gao *et al*.,2016) and there is large number of subtypes evolved within each serotype (Ateya *et al*.,2017). FMD is the most contagious viral disease of animals and endemic in many parts of the world, including most parts of Africa, Asia and some parts of South America. In FMD-endemic regions the major economic loss of the disease is associated with reduced livestock productivity, regular mass vaccination and trade restrictions on animals and livestock products (Niedbalski *et al*., 2003). Four serotypes (A, O, SAT 1, and SAT 2) have been reported over the past ten years in Ethiopia. Sero-type O and A are the dominant serotypes out of for serotype reported in Ethiopia (Endris *et al*., 2018); while serotype C was last reported in 1983 (Sulayeman *et al*., 2018). In Ethiopia, factors such as susceptibility of animals, wild and domestic animals indirect contact on the grazing pastures and watering points and uncontrollable animal movement within the country and transboundary contribute to the frequent occurrence of FMD outbreaks and to the difficulty in controlling the disease (Admassu *et al*., 2015).

The properties of antibodies to recognize small specific structures on the antigen to which it induced by immune cell of the animals were made them an important tool in laboratory for various applications of diagnosis (Chalghoumi *et al*., 2009, Ateya *et al*.,2017, Ibrahim *et al.*, 2017). the use of chickens instead of mammals for polyclonal antibody production has increased in recent times. A major advantage of using birds in the antibodies production is that the antibody can be harvested from the egg yolk instead of serum, thus making reduce the stress and repeat bleeding of the animals and the antibody productivity of an egg-laying hen is much greater than that of a similar sized mammal (Hau *et al.*, 2005, Fischer *et al*., 2014), however, the recent technologies allowed the development of an alternative (egg yolk) method to the conventionally serum-based techniques (Ahmad *et al*., 2017, Khan *et al*., 2017). An outstanding advantage of IgY-Ab is specificities to mammalian antigen, animal welfare since the Ab are non-invasively extracted from egg yolk, high ab per ml, very cost-effective (Pauly *et al*., 2011) and short turnover time from immunization of an immunogen to the isolation of an antiserum (Kousted *et al*., 2017). Chicken egg yolk antibodies (IgY) have major importance as a safe and low-cost source of specific antibodies in previous 20 years (Antonysamy *et al*., 2018). Using chicken as a host for generation of polyclonal antibodies (IgY) has valuable advantages over existing mammalian systems, despite it is still an underused resource in Ethiopia. Therefore, most of the previous antibody production in Ethiopia uses laboratory animals such: as mice, rabbit, guinea pigs and other mammalian, but the current study was designed to produce antibodies of selected FMDV serotypes (A, O and SAT-2) in the way that ensure animal welfare only by immunizing layer hens and provide a specific IgY used for diagnosis and vaccination of selected FMDV serotypes from egg yolk.

Therefore, the objective of this study was:

The General objective

- ❖ To produce Foot-and-mouth disease (Serotype: O, A and SAT-2) polyclonal antibodies (IgY) by using layer hens.

Specific objectives

- ➢ To detect and evaluate the antibody titer in sera and egg yolk sample against the FMDV.

2 LITERATURE REVIEW

2.1 Introduction

Foot and Mouth Disease (FMD) is the contagious viral disease of mammals and causing severe economic loss in susceptible cloven-hoofed animals. It is categorized as list “A” disease according to OIE disease classifications. The disease was identified for the first time by Friedrich Loefffler in 1898 (Admassu *et al*., 2015). It is characterized by high morbidity, vesicle formation and erosion in the mucosa of the mouth, nose, and interdigital space (Wungak *et al*., 2017). At early stage of FMDV infection, the innate immune system of the host animal is activated to form an antiviral state. The innate immune response provides the first line of defense, which is crucial for preventing infection (Zhang *et al*., 2018). The virus elicits a rapid humoral response in both infected and vaccinated animals. The response is directed to epitopes on the three external structural proteins and good protective immunity is established between 7 and 14 days after e infection or vaccination. Clearing the virus from the infected animal by phagocytosis of opsonized virus carried out by Macrophage (Ibrahim *et al*.,2018). protective immunity to FMDV is mostly due to neutralizing antibodies and T-cell response (Blanco *et al*.,2013). inactivated FMDV Vaccination within aqueous aluminum hydroxide and saponins or oil based adjuvants, play an important role in control measure of FMDV (Jouneau *et al*., 2020). Layer hens are a desired source of antibodies, represent the best alternate animal system antibody production as it offers some advantages such as: care to animals, high productivity and special suitability of avian antibodies for certain diagnostic purposes. However, it’s an excellent counterpart to mammal IgG chicken, IgY antibodies were underused resource; due to the lack of information concerning the possibility of production and application of IgY or their use is hindered by problems with keeping the chickens and with IgY isolation (Narat.2003a).

2.2 Etiology

Foot and mouth disease (FMDV) are classified within the *Aphtho* virus genus as a member of Picornaviridae family and exists in seven serologically distinct serotypes: O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. based on the sequence of the viral structural protein gene (VP1), which reflects genetic, antigenic and geographical relationships among the strains, each serotypes are further delineated into clades (topo type) (Khodary *et al*.,2018).within each serotype, there are a large number of strains with their own antigenic characteristics, so there may be only partial cross-immunity between strains of the same serotype (Shawky *et al.*,2016).

2.2.1 Structure of FMDV

Foot and mouth disease virus is a small, non-enveloped and single standard positive sense RNA virus (Ateya *et al.*,2017), which serves as both mRNA and the template for negative-stranded RNA. The FMDV particle is roughly spherical (Icosahedral) in shape and about 25–30 nm in diameter. It consists of the RNA genome surrounded by a protein shell or capsid. The capsid is composed of 60 copies of the capsomers. Each capsomer consists of four structural polypeptides, VP1, VP2, VP3 and VP4. The VP1, VP2 and VP3 are exposed on the surface of the virus while VP4 is located internally (Jamal *et al*., 2013, Belsham *et al*.,2004).The genomic size of virus varies according to the serotype, but it is estimated at about 8.5Kb and as the following 3 components: a 5′-untranslated region (5′- UTR), an open reading fragment (ORF), and a 3′-UTR. The ORF encodes a polyprotein that forms four fragments after the primary cleavage: The L fragment, the P1/2A fragment, the P2BC fragment, and the P3 fragment. After the secondary cleavage and the maturation cleavage, 4 structural proteins (VP4, VP2, VP3, and VP1) and 8–9 non-structural proteins (Lab/Lb, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D) are formed (Wang *et al*.,2015).

The 5' UTR contain 1300 nucleotides while 3' UTR have 100 nucleotides. The viral genome replication is mainly occurred under 5' UTR and it also initiates the viral polyprotein by using cap independent translation. The 3' UTR have 90 nucleotides in length and also involved in genome replication through its cis-acting elements (Naveed *et al*.,2018). Also, secondary structural element of 5'UTR consist of: the short fragment (S-fragment), poly-c tract, RNA pseudoknots, the cis-acting replication element (cre) and internal ribosome entry site (IRES). From 5'UTR secondary structure elements, the cre and IRES play an important for translation and replication. The 3'UTR also contains secondary structure elements. There are two stem loops directly following the ORF called stem loop 1 (SL1) and stem loop 2 (SL2) which are involved in viral replication. The 3' end of the genome consists of a poly-A tail (Logan *et al.*,2017). Primary co-translational processing of P1-2A, P2, and P3 precursors are formed by the cleavage of the leader protease (Lpro), 3Cpro and a translational recoding event mediated by 2A. Lpro, a papain-like cysteine proteinase, is formed in two unique forms: Labpro and Lbpro, which generated by translation initiation at two in-frame AUG codons separated by 84 nt on the viral RNA and subsequent intra-molecular self-processing (Pulido *et al.*, 2017). Two virus-encoded proteinases (leader (Lpro) and 3Cpro) form structural and NSPs protein by cleavage of polyprotein of about 250 kDa which formed from translation of viral ORF. Maturation of polypeptides have different functions which form four functional regions of ORF in FMDV genome as (**Figure 1**): L region, which is located at 5′ end and codes for Lpro. P1 region, encoding a precursor for capsid polypeptide (VP4, VP2, VP3, and VP1). P2 region encodes (2A, 2B, and 2C) And P3 region (3A, 3B, 3Cpro and 3Dpol, in which, 3C is a viral protease and 3D an RNA-dependent RNA polymerase) (Gao *et al.*, 2016).

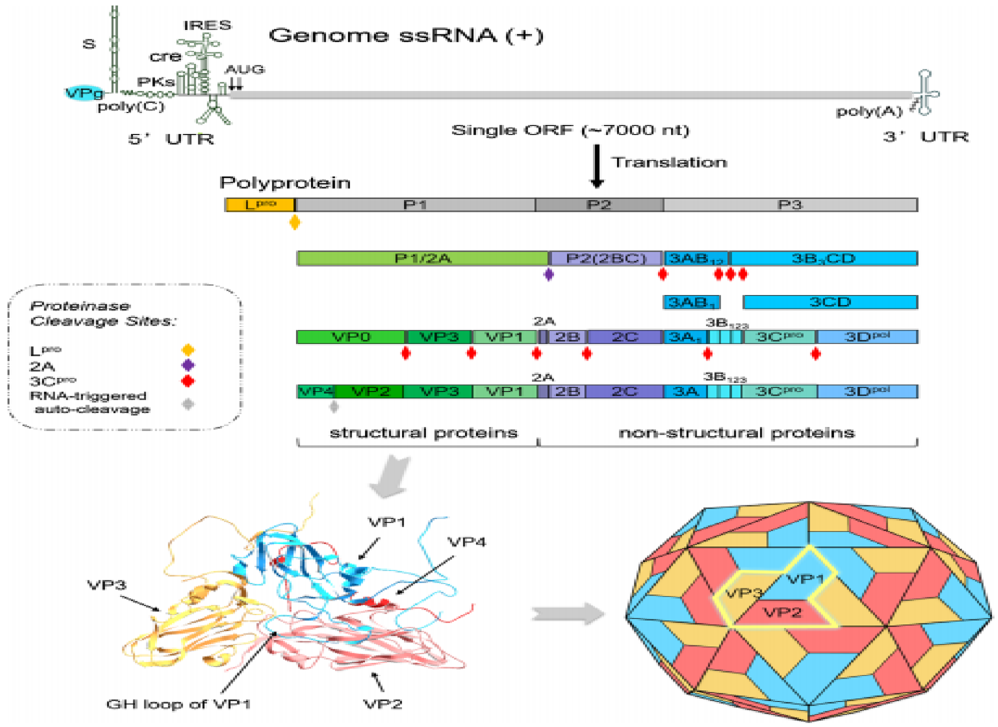

Figure 1: Schematic diagram of FMDV genome, processing of viral polypeptide and conformations of the structural proteins(Gao et al., 2016).

2.2.2 Antigenic Variation

To evade a host immune response, an infectious organism change structure and function of its surface proteins by amino acid substitution, this process is called Antigenic variation. These make an organism long-lived in host, repeatedly infect a single host, and are easily transmitted. One main character of RNA viruses is an error-prone RNA replication, which results variation of an organism (Neeta *et al*., 2011). Such like Antigenic and genetic diversity hinder eradication of FMDV through vaccination. Antigenicity is mainly expressed by the surface coating proteins (capsid). These antigenic variability classified FMDV in to Seven immunologically distinct serological types of FMDV namely: serotypes O, A, C, Asia 1 and SAT (Southern African Territories) 1-3 (Chakraborty *et al*.,2014).

The whole genome FMDV serotypes have an average of 86% nucleotide sequence identity to each other across, While the VP1 coding region is more variable with about 50-70% nucleotide sequence identity (Jamal *et al*.,2013). Antigenic variation in FMDV serotypes is caused by VP4.

The binding of the Arg-Gly-Asp motif with recogVP1, VP2, and VP3 that have structural and sequence similarity results the viral antigenicity. Most serotypes of FMDV share high variable sites of VP1 with 135-155 amino acid residue. High sequence variability in Beta-cell epitopes cause low cross-reactivity between the serotypes of FMDV. These regions also contain antigenic site "A" in which the high mutation in RNA replication occur (Naveed *et al*.,2018).To determining the antigenic properties of the virus, the surface-exposed capsid proteins of FMDV are also critical for binding of the virus to cells (Gullberg *et al*.,2019).

2.3 Epidemiology

Currently, in Asia, Africa, and Venezuela in South America, FMDV is endemic (Jouneau *et al*., 2020. Herieth *et al*., 2016). Host species, population density, animal movements within and transboundary and indirect contacts between domestic and wild host species are the main factor for the multiplication and transmission of FMDV. The environment become the geographical barriers to virus dissemination. this multi factorial scenario, results FMDV variation and adaptation has been modeled as a complex evolutionary patterns that can revealed by molecular epidemiology analyses or nucleotide sequencing of capsid protein genes (Longjam *et al*., 2011). VP1 coding nucleotide sequences can indicate a tendency for same viruses to re-emerge in the same geographical area or another new virus to the given geographical area. There is a genetically and antigenically distinguishable, seven FMDV regional pools, however some countries share viruses belonging to two different pools (**Figure 2**) (Jamal *et al*., 2013).

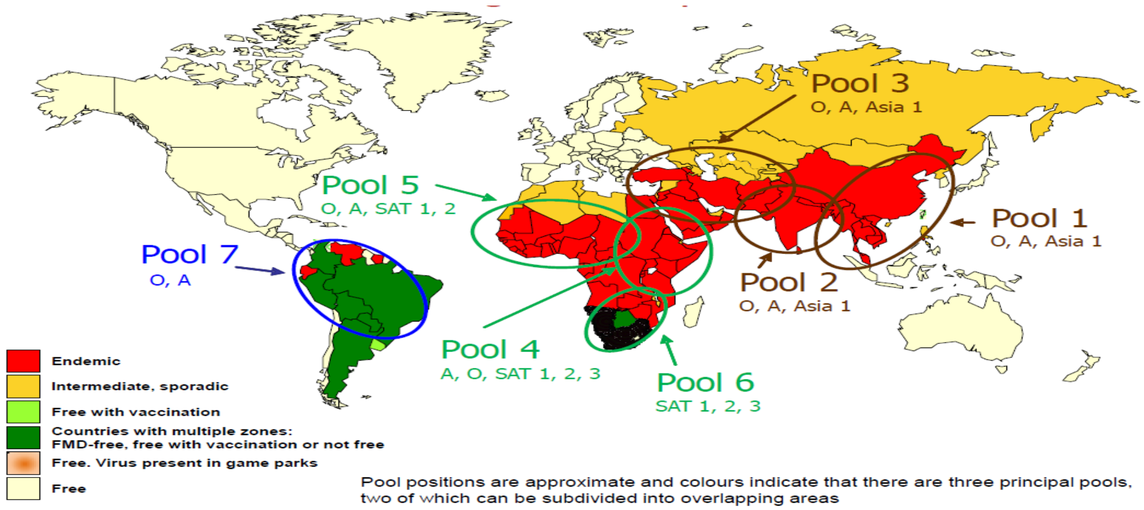

Figure 2: *Geographical pools of FMDV (source: Robinson* et al.*, 2014)*.

Six serotypes of FMDV (O, A, C, SAT-1, SAT-2 and SAT-3) have been known in African countries, while four serotypes in Asia (O, A, C, Asia-1) and three in South America (O, A, C). Even if FMDV classified in seven pool, SAT-1 and SAT-2 serotype spread from Africa to the Middle East. Globalization make the spread of FMDV worldwide (Aftosa *et al.*, 2015). Commonly, east African have been affected by FMDV serotype O, serotype A and SAT2 respectively (jones *et al*., 2017).

2.4 Pathogenesis

All cloven-hoofed domestic and wild animal can be infected by the FMD virus, however the development of the disease is depending the host and virus (Admassu *et al.,* 2015). The most important portal of infection of FMDV is respiratory system. The virus can affect the pharynx and primary multiplication of the virus is occur on the mucous membrane and by lymphatic and blood circulation transported to the sites of secondary multiplication in the lymphatic glands, epithelial tissues in and around the mouth, feet and in the mammary glands. Before the appearance of clinical signs of FMD following secondary replication in other glandular tissues, the FMDV can be detected in body fluids such as: milk, urine, respiratory secretions and semen, the virus can also persist for long periods after the acute infection. In cattle, virus may be detectable up to 2 years periods after exposure to infection and up to 6 months in sheep. Gross lesions develop only in areas exposed to mechanical trauma or unusual physiological conditions such as the epithelium of the mouth, feet to a less extent, the teats.(Admassu *et al*.,2015).The dorsal surface of the soft palate and the roof of the pharynx, just above the soft palate, are sites of particular significance. Viral symptoms occurs subsequently within the cornified stratified squamous epithelia of the skin (including the feet and mammary gland) and mouth (including the tongue) and myocardium of young animals (Alexanderson *et al*.,2003). The severity of FMDV clinical signs on cattle and pigs is highly sever, while that of sheep and goat is discrete (Jouneau *et al*.,2020). The replication of the virus is rapid after entry through the upper respiratory tract or lung, viremia seeding infection into the epithelium where secondary virus multiplication results in vesicles and shedding from the udder in milk (Ateya *et al*.,2017).

The severity of FMD is high in young animals, with a higher mortality rate due to myocardium degeneration, whereas adult animals generally clear infection within two weeks. The virulence determinants in FMDV is the viral leader protease, which inhibits induction of beta interferon mRNA and blocks the innate immunity of the host animal (Kamel *et al*., 2019). FMD pathogenesis are variably species-specific (Arzt *et al*.,2017).

2.4.1 Genome Penetration

Integrin are heterodimer proteins composed of two differing subunit, type 1 trans membrane subunits, a and b, with large extracellular domains and usually a cell entry determinants short cytoplasmic tail of FMDV (Stuart *et al.*,2005). Many evidences show that conformational changes result in the insertion of VP4 and the N-terminus of VP1 into the cellular membranes to form the channels through which the genomic RNA might traverse the membrane. An acid condition of host cell results the dissociation of the virus into pentameric subunits, VP4 and the genome. Then after the RNA enter into the cytoplasm, while the capsid proteins left in cell endosomes. It is possible that VP4 may associate with the endosomal membrane to produce channels into the cytoplasm (Stuart *et al.,2005)*. The following figure (3) shows the viral genome (A), replication cycle(B) of virus in to the host cells.

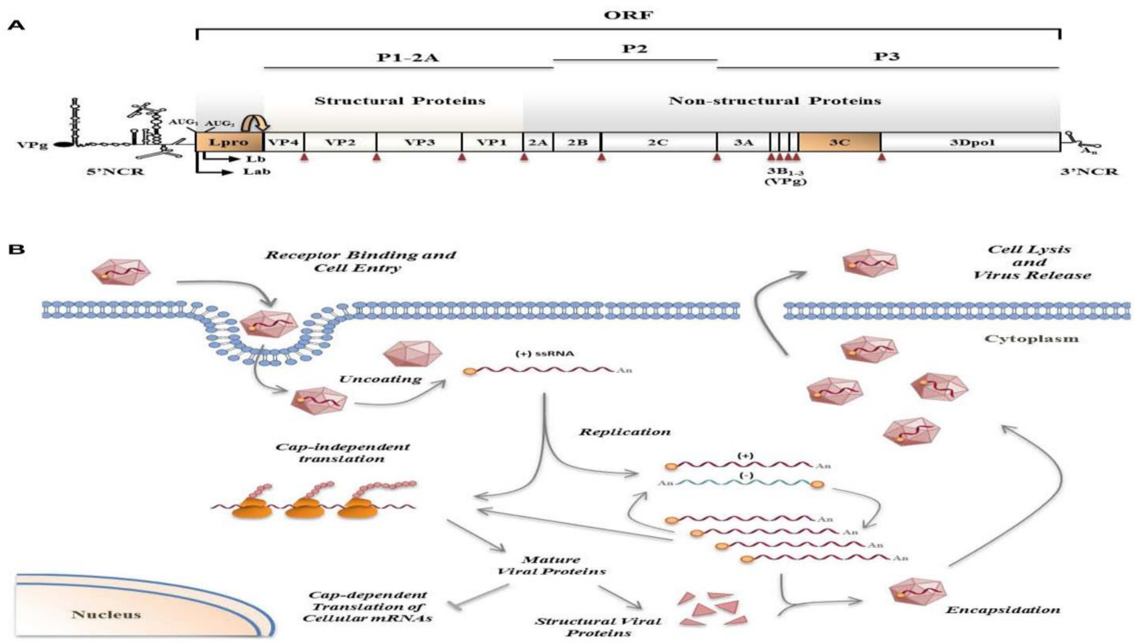

Figure 3: FMDV genome (A) and viral life cycle (B)(Pulido et al., 2017)

All FMDV replication cycle occurs in the cytoplasm. The information required to inhibit the host cellular machinery and induce the shut-off the host macromolecular synthesis in infected cells is carried on viral RNA. The positive-strand RNA transcribed into a complementary, negative-strand RNA molecule by the RNA-dependent RNA polymerase. Then, 3Dpol generates multiple positive-strand RNAs which either enter a new round of translation and RNA replication, or are packaged by the capsid proteins to form new virus particles which are finally released by cell lysis (Pulido *et al*., 2017).

2.4.2 FMDV life cycle

After entry through the upper respiratory tract or lung, the virus is rapidly replicated. The virus shed from an infected animal through host secretions and excretions and cause infection across damaged epithelium (Ateya *et al*.,2017). First replicates of virus occur in the pharynx. In 24–48 h the virus spread across the blood stream and lesions appear in the mouth and feet of susceptible animals. However, the Viremia disappears after 3–4 days, virus replicates to very high titers (>8 log 10 infectious units per ml) at lesions sites and shed in the air and body fluids. The virus establishes persistence in the pharyngeal region of approximately half of the infected animals, even vaccinated animals protected from clinical disease, that become long-term carriers (Rodriguez *et al*.,2009). The other viral NS proteins are involved in various aspects of the viral replication cycle. 3D is the viral RNA-dependent RNA polymerase, 3B (also termed VPg), a protein covalently linked to the 50 end of virions RNA, has a role in the initiation of RNA synthesis, and 2B, 2C, and 3A are involved in membrane rearrangements required for viral RNA replication and capsid assembly (Grubman *et al*.,2008).

2.4.3 Clinical signs of FMDV

There is some variability in the clinical signs among species. Foot-and-mouth disease is typically an acute febrile illness with vesicles on the feet in and around the mouth and the mammary gland. Vesicles occur occasionally at other locations, including the vulva, prepuce, or pressure points on the legs and other sites. The vesicles usually rupture rapidly, becoming erosions. Pain and discomfort from the lesions lead to clinical signs such as depression, anorexia, excessive salivation, lameness and reluctance to move or rise. In serious cases, the hooves or footpads may be sloughed. Reproductive losses are possible, particularly in sheep and goats. Deaths are uncommon except in young animals (Naveed *et al*.,2018).

2.4.4 Routes of Transmission of FMDV

Spread of FMD virus is most commonly associated with the movement of infected animals and their contact with susceptible animals. During the early stages of disease, infected animals shed virus in all their excretions and secretions, including their breath. In cattle and pigs peak production of virus coincides with the onset of clinical signs, whereas in sheep it occurs before the appearance of lesions, and then in all species it declines rapidly as antibody production and other immune responses bring the infection under control. FMD virus can also be carried mechanically by people, vehicles, brushes, surgical equipment and other fomites from infected to susceptible animals (Kitching .2005). 44% of FMDV transmission is airborne or environmental transmission. Its travel over water is estimated at maximum distance of 250kms. The intensity of the disease is determined by the. Dose of virus ,Species of the sick animal and the susceptible host (Towhid et al. 2016)..

2.4.5 Incubation period of FMDV

The incubation period of FMDV from farm to farm is in 4 to 14 days range of spread, while it's in range of 2 to 14 days within the farms even if it may be as short as 24 h in pigs and in highly dense population. The incubation period for FMD is highly variable depends on: the strain and dose of virus, route of infection, species of host and the husbandry conditions of animals (Alexanderson *et al*., 2003).

2.4.6 Excretion of Virus by Infected Animals

The virus may secreted and excreted from an infected animal occur through: urine, milk nasal droplet, damaged epithelium and orally (Ateya *et al*..2017)**.** The animal body secretion and excretion may contain a significant titer of virus before the development of clinical signs, particularly in saliva, nasal and lachrymal fluid, milk, but Urine and feces contain virus in the lesser extent. Since preputial lesions are sometimes present it is possible that these are the origin of infectivity in urine (Alexandersen *et al*., 2003).

2.4.7 Carrier State

FMD viruses persisted in naso-pharynx of chronically infected animals (Thomson *et al*.,2011).During the first three months after infection of FMD small amounts of FMDV may persist in the throat, which may reach 50% in recovered cattle. However, the number virus in infected anima is decreases with time, the small percentage of virus remaining at two years post infection. In Vaccinated animal which may exposed to FMD virus, the virus can persist for longtime without showing clinical signs. FMD virus is found only in small quantities in the pharyngeal area of carriers(IAEA, 2007). Most of the time the sheep shows mild or no clear clinical signs of FMD, however they can secrete and excrete certain amounts of FMDV as a its carrier (Abd *et al.,* 2015).

2.5 Immunoglobulin

An immune response is established in animals to foreign substances (antigens), which induce *anti*-body production. Collectively, immune response is produced by innate immunity which clear a pathogen and adaptive immunity with the memory. The basic component of immune response includes: immune cells, cytokines, and chemokine's (Shah *et al*.,2015). Antibodies is macromolecule that recognize foreign molecules in the host system. Foreign substance which cause the host body to mount an immune response are called antigens. Antigen may include infectious agents like: bacteria, viruses, fungi and other foreign substance. Antibodies can make the foreign particles recognizable to other cells of the immune system by its ability to specificity to locate and attach themselves to their targeted foreign substance (Veerasami *et al*.,2008). Antibodies play an important role are in prevention and resolution of infection by foreign invaders (Kumagai *et al.*, 2001). (Kumaran *et al*., 2018). Mammalian immunoglobulin is classified into five class: IgG, IgM, IgA, IgD, and IgE. These immunoglobulins differ in size, charge, carbohydrate contents, and amino acid composition (Buyukkoroglu *et al*., 2018). Mammalian embryos accepted as "self "all maternal foreign proteins, but the neonatal immune system start to matures and learns to differentiate between "self" and "oneself immediately after its birth (Burns *et al.*,2005).

2.5.1 Immune response of foot-and-mouth disease virus infection

The protective immune response against foot-and-mouth disease (FMD) virus is demonstrated as depend on the interaction between virus specific antibody and the phagocytic ceils of the reticuloendothelial system (Mccullough *et al*., 1991). VP1 carries important immunogenic sites recognized by host immune cells, including amino acids (aa) 141–160, a major B cell site, and aa21–40, a T-cell site (Li *et al*.,2004). Pega et al. report that, FMDV-specific antibody secreting cells (early B-cell response to aerosol infection) are established in the lymphoid tissue of the respiratory tract and spleen after four days of infection. The same group has extended this work to show that systemic vaccination is able to induce a B-cell response which extends to the lymph nodes draining the respiratory system, and can result in a rapid recall response in the respiratory mucosa upon aerosol exposure to live virus (Robinson *et al*., 2016).

FMDV disease rapidly induce strong and long-lasting antibody against the FMDV antigen in susceptible hosts. Following the first detection of antibody in host, the virus can be cleared from blood and lymphatic circulation and the virus shedding from host secretion can be reduced gradually. Although the circulated antibodies are expected prevent viremia and generalized

FMDV disease, it does not prevent localized virus in organ such as pharynx (Eschbaumer *et al.*,2016). Recognition receptors in the host cell can recognize the virus-associated molecular patterns upon the infection of the host. The recognition of virus-associated molecular receptors causes interferon type 1 (type 1 IFN) and interferon-stimulated genes (ISGs) to be transcribed. The down regulation of interferons is a stimulatory factor for enhanced expression of natural killer cells; thus, an antiviral state is established in the host (Naveed *et al.*,2018).

FMDV have the FLAG epitopes polypeptide with Arg-Gly- Asp (RGD) tripeptide sequence motifs. The animal did not produce antibodies against FLAG as it remained cryptic site and not detected by the immune system (Naveed *et al.*,2018). However, adjuvant boosted inactivated virus (FMDV) vaccine induce effective protective immunity related antibody (Ab) responses (Jouneau *et al*., 2020), vaccination with one serotype does not provoke immune protection to the other serotypes (Wungak *et al*.,2017). The poor antibody responses resulted in calves and piglet's due interference by colostrum and immaturity of its immunity. The antibody response to FMDV is T cell dependent. different species of animal recognize different epitopes as different T cell recognition sites, therefore, FMDV antigens are not equally immunogenic in different species (Thomson *et al.*,2011). Also low cross-reactivity are due to high sequence variability in Beta-cell epitopes (Naveed *et al*.,2018). In vaccinated animals' induction of cell-mediated immune responses low as comparing with naturally infected animal as a result of the NSP removed from viral genome of inactivated vaccine viruses. In both the capsid proteins and NSP of viruses, helper T-cells (Th) recognize epitopes which are presented by Dendritic cells as it respond to B-cells, However, NSP removed from in inactivated vaccine, the T-cell responses is induced by CD4+ cells which contribute in the production of FMDV antibodies. (Park *et al*., 2013). The potential importance of non-neutralizing opsonic antibodies that facilitate uptake of bound FMDV by dendritic cells (DC), which are potent immune modulators. Opsonizing antibodies increased plasmacytoid DC-mediated release of antiviral Type I IFNs in response to both homologous and heterologous serotypes (Robinson *et al.*,2016).

The viral leader proteinase, Lpro, limits the host innate response by inhibiting the induction of interferon beta (IFNb) mRNA and blocking host cell translation. A second viral proteinase, 3Cpro, may affect host cell transcription because it cleaves histone H3. Viral protein 2B in conjunction with 2C or their precursor 2BC inhibits protein trafficking through the endoplasmic reticulum and Golgi apparatus. A decrease in surface expression of major histocompatibility class I molecules during FMDV infection suggests that 2B, 2C and/or 2BC may be involved in delaying the initiation of the host adaptive immune response and also adversely affect the secretion of induced signaling molecules (Grubman *et al.*,2008). High IFN activity is detectable in tissues of FMDV-infected animals, but it is restricted to sites of virus replication (Eschbaumer *et al*.,2016).

2.5.1.1 Neutralizing antibody of FMDV

Innate immunity plays an important role in defending a host from invading pathogens. Type-I IFNs, such as IFN-a and -b, are key players in the innate antiviral response(Li *et al*.,2016). B-cell induce the response by T-cell help or in the absence of T-cell help by type II T-cell independent (TI-2) antigens, such as viral capsids, that have regular repeating epitopes within their structure (Grant *et al*.,2016). The specific site for binding to neutralizing antibody of FMD capsid is the G-H loop in the VP 1 peptide. B lymphocytes recognizing epitopes on the virus particles to produce specific antibody following antigen processing and presentation by the major histocompatibility complex type II, the growth and differentiation factors necessary for developing the immune response.is resulted from stimulation of the T-helper (Th) lymphocytes (Motamedi-Sedeh *et al*., 2015). Production of virus-antibody complexes early in the infection stimulating the production of interferon and consequently stopping the production of virus within the cells. Increasing this parameter therefore produces virus-antibody complexes sooner and stops the production of virus in the cells sooner (Howey *et al*.,2012).

2.5.1.2 Integrin receptors of host

For its entry to the host cell, The FMDV has the Arg-Gly-Asp (RGD) ligand which makes its complementary with integrin receptors of host cells. Amino acid sequence at residues about 141–160 of VP1 in the O serotype, which corresponds to the G-H loop, contain the most immunogenic site and RGD ligand (Cho *et al*.,2012). The interaction of host cell receptor (integrin αvβ6) with the FMDV is determined by cation. Type -I interferon is induced in cultured cells and experiments by the FMDV 28 non-coding RNA fragments (Website 2010). Induction of type I interferon (IFN-α/β) mRNA is the first responses of the host to viral infection during host viral interactions. Expression, secretion, and binding of IFN-α/β protein to specific receptors on cells results in initiation of a signal transduction pathway and induction of a virus-resistant state in these cells by activation of a series of genes whose protein products can inhibit various steps in the virus life cycle (Grubman 2006)

2.5.2 Adjuvants

To generate an antibody against a protein of interest, the standard approach is to inject small samples of the protein (in the microgram range) into an animal such as a rabbit. However, administration of antigen alone is rarely sufficient to provoke a robust immune response, even if the antigen is composed of a high proportion of non-self-determinants; co-administration of an adjuvant is required. While it is not entirely clear exactly how adjuvants work, one important role they perform is to activate dendritic cells (DCs) and other antigen-presenting cells (APCs) at the site of antigen delivery .(Petter *et al.*, 2017). One classic mechanism of adjuvant action is the "depot" effect, in which the adjuvant protects the antigen from both dilution and rapid degradation and elimination by the host. The adjuvant permits a slow, prolonged exposure of the immune system cells to a low level of antigen by localizing and slowly releasing intact antigen. This prolonged exposure results in continued stimulation of antibody producing cells, resulting in the production of high levels of antibody by the host (Stills *et al.*,2005).

The ratio of the aqueous antigen to the oil adjuvant was 50:50 for all the current vaccine batches (Fawzy *et al.*,2015). Al(OH)3-saponin adjuvant FMD vaccines are significantly used in cattle, sheep and goats, but poorly effective in pigs, however the oil-adjuvant vaccines can be used in all species (Sebhatu *et al.,* 2014).These oil adjuvant have ability to induce a rapid, high and long-lasting immune response in all animal species. (Ibrahim *et a*l.,2015). Gel- or oil-adjuvants are commonly used for an Inactivated FMD vaccines. Gel vaccines are only used in cattle; however, recently oil-based adjuvants are used in the worldwide as it improving the level of immunity induced and long-lasting antibody. In vaccinated animal cell-mediated immune response is induced by Water in oil(W/O) ,while humoral immunity are boosted by water- oil –water(W/O/W) and oil –water (O/W) (Park *et al*., 2013).

2.5.3 Avian immune system

There are two classification of avian lymphoid organ of birds: The primary lymphoid organs and secondary lymphoid organs play a significant role in avian immune systems. The Primary lymphatic organs of the avian are thymus, which located in the neck region along the jugular vein, and the bursa of Fabricius is located nearby to the cloaca. Secondary lymphoid organs of the avian includes: Harderian gland, bone marrow, caecal tonsils, spleen and also there is organized mucosal surfaces associated lymphoid tissues (MALT) of the bronchial associated lymphoid tissues (BALT), gut associated lymphoid tissues (GALT), conjunctival associated lymphoid tissues (CALT) and lymphoid nodules. There is also a lymphatic circulatory system of vessels and capillaries that transport lymph fluid through the bird's body and communicate with the blood supply (Chalghoumi *et a*l., 2009). The chicken has significant importance model in the area of developmental biology and immunology (Schusser *et al*.,2013). The IgY produced by the hyper immune chicken egg yolk are an excellent alternative source of Polyclonal antibody (Veerasami *et al.*,2008).

2.5.4 Hen Egg Yolk Antibodies

IgA, IgM, and IgY are three immunoglobulin classes, which distinguishable in concentration, structure, and immuno- chemical function, are found in birds. The IgA and IgM are similar to mammalian IgA and IgM in molecular weight, structure and electrophoretic mobility (Chalghoumi *et al*.,2009). IgY is the predominant serum Ig of the chicken and is the evolutionary ancestor of both IgG and IgE (Ulmer-franco *et al*., 2012). IgG and IgY are functionally equivalent to each other and interchanged (Antonysamy *et al*.,2018). IgY in chicken and IgG in mammals are structurally different in their: molecular weight ,absence of the hinge region in IgY and one heavy chain they possess an additional highly glycosylated constant domain in IgY (Hodek *et al*.,2013,Fischer *et al*., 2014). chickens antibody production can solve the problem with the painful and stressful collection of blood serum and reduce the number of animals used in designed experiment as chickens produce larger amounts of antibodies per egg and hens (Narat .,2003).

Chicken IgY is mainly in systemic rather than a secretory antibody system. also IgY is found in duodenal contents, tracheal washings, seminal plasma and egg yolk (Fischer *et al.*, 2014). Currently, the chicken egg yolk antibody (IgY) get more attention as it can be prepared easily, in high concentration, affordable and safe methods. New vaccine technology has led to vaccines containing highly purified antigens with improved tolerability and safety profiles, but the immune response they induce is suboptimal without the help of adjuvants (Kumaran *et al.*,2016).

2.5.4.1 Advantage of Hen immunoglobulin

This immune globulin can have broad applications from developing immunoassays to treating disease. Antibody production in eggs is particularly advantageous because hens can be effectively immunized, antibodies are readily deposited in the yolk, and eggs are a convenient and inexpensive (Chalghoumi *et al.*,2009 and Lloyd-Jones *et al.*,2017). Using chicken as the immunization host for producing egg yolk antibodies (IgY) have significant advantages incudes: antibody isolation is fast and simple, reduced animal suffering(no bleeding),; very low quantities of antigen are required to induce immune response in the immunized hens, one egg contains as much antibodies as an average bleed from a rabbit (Ibrahim *et al.*,2017), there is no interference with mammalian IgG, and do not activate mammalian complement (Chalghoumi *et al.*,2009). The phylogenetic distance and genetic difference of IgY from mammalian make to develop highly specific antibodies against conserved mammalian proteins. IgY and IgG does not crossly react with each other, rheumatoid factor, human anti-mice antibodies, and heterogeneity glycoproteins. Therefore, IgY have unique features useful for different immunology and diagnostic technique with low of false-positive results. Low quantity of antigen is required to induce a humoral response with 100 mg of IgY per egg yolk. IgY technology is both readily available and economical(Khan *et al.*,2017). PAbs has some problems of batch to batch variation, inconsistent yields of antibodies and scanty serum obtained from individual animals. The IgY derived from hyper immune chicken egg yolk has been recognized as an excellent, alternative source of PAbs (Veerasami *et al.*,2008).

2.5.4.2 Structure of Hens antibodies

Immunoglobulin's IgY are commonly found in the serum of birds, reptiles, and amphibians, and transferred from blood (serum) to egg yolk as a maternal immunity to provide the passive immunity to their embryos and neonate(Ibrahim *et al.*,2018). It is the major antibody isotype in the chicken serum and is composed of two heavy and two light chains, each of them with molecular mass of 67 to 70 and 25 kDa, respectively and as it has one additional constant region in heavy chains, its molecular mass (~ 180 kDa) is higher than mammalian IgG (Raj *et al.*,2004,Reza *et al.*,2018). In eggs, IgY is present predominantly in the egg yolk whereas IgA and IgM are present in the egg white as a result of mucosal secretion in the oviduct (Length *et al.*,2014). Because IgY-Fc has an amino acid sequence different from IgG-Fc, IgY structures do not have protein A and G binding specificity, which have significant importance for mammalian antibody affinity purification (Khan *et al*.,2017).

IgY antibodies are differentiated from IgG in structural and functional characteristics. The main difference is: the number of constant regions (C) in H chains, a greater molecular mass of IgY compared to IgG *i.e.* 180 and 150 kDa respectively, the absence of the hinge between C_1 and C_2, make IgY is much less flexible than IgG (Narat .,2003), additional evolutionary precursor to the mammalian IgG hinge region domain (CH2) and its capable of mediate anaphylactic reactions function as IgE in mammals (Fischer *et al*.,2014).

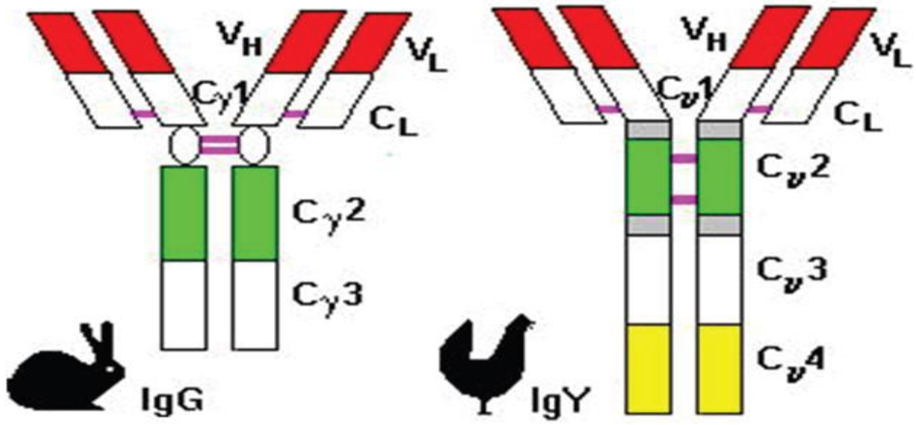

Figure 4: Structural differences between mammalian IgG and chicken IgY (Ulmer-franco *et a*l., 2012).

2.5.4.3 IgY use for passive immunization

Immunity against foreign body can be transferred naturally as active humoral immunity (ready-made antibodies) from maternal antibodies to the offspring via placenta. It may be induced when animal are exposed to specific antibodies for particular pathogen, a toxin or from patient recovering from the infection and administered to non-immune individual. The antibodies transfer may be carried out via systemic, intravenous or oral route (Chalghoumi *et al*.,2009).

The immune system of a newly hatched chick is not fully developed and antigen-specific protection must be received from maternal antibodies. Contrary to mammals that provide maternal antibodies to their offspring via the placenta or the colostrum, hens must deposit maternal antibodies in the egg before it is laid. In chickens, the transfer of IgY from the hen to the chicks is a two-step process. The first step involves: selective, receptor-mediated transferring IgY from the bloodstream of the hen into the ovarian follicle, and the second, transporting IgY from the egg yolk to the embryo. Absorption of yolk IgY via the yolk sac slowly begins around 7 days of incubation followed by embryonic uptake through hepatic and portal circulation. After perforation of the sero-amniotic connection unaltered proteins present in the albumen move into the amniotic fluid and are swallowed by the embryo (Ulmer-franco *et al*., 2012). There is a delay of five to six days since the appearance of IgY in serum until it is found in the yolk. The amount of IgY in the yolk is equal to the IgY concentrations in serum. However, the amount of IgY in egg yolk varies greatly between different bird species and even between chicken lines, the Yolk IgY concentrations of the IgY is 7.9mg/ml to 20-25mg/ml in ranges (Fischer *et al.*,2014).

2.5.4.4 IgY production

Chickens can be used for antibody production throughout their egg laying period(Fischer *et al*.,2014).To generate specific IgY antibodies to an antigen of interest, laying hens must be immunized with the target antigen. By using gene expression vectors, hens can be immunized with a specific target gene for the production of a gene-specific IgY antibody (Lanzarini *et al.,*2018). Immunoglobulin (IgY) of the egg yolk antibodies is used as a good option for large-scale production of polyclonal antibodies in economical ways. Chicken IgY are transferred from blood to the egg yolk during embryo development (Zhu *et al*.**,** 2019) Specifically IgY production can be achieved by immunizing layer hens with the target antigen. However, the resulting immune response of the immunized hens cannot be very predictable (Kumaran *et al*.,2018), the production and amount of IgY produced may affected by the antigen used, breed, age, adjuvants, route of immunization, maintenance, climatic conditions, chronobiologic impacts and so on (Antonysamy *et al*.,2018).

2.5.4.5 IgY extraction

Various economical, simple and highly effective techniques for IgY extraction such as: water dilution, polyethylene glycol or ammonium sulfate precipitation, gel filtration, thiophilic gel chromatography, ultrafiltration and ion exchange chromatography can be used for the isolating and purifying IgY from egg yolks. However, most previous IgY extraction techniques involve changing temperature, ion strength, and pH, which are non-specific and time-consuming processes that achieve less than 50% yield and less than 80% purity (Khan *et al*.,2017, Ahmad *et al.*,2017).

The purification of IgY from separated yolks usually proceeds in two stages: in the first one, the water-soluble fraction (WS) containing IgY is separated from the lipid fraction composed mainly of lipids and lipoproteins, and in the second stage, IgY are isolated from the other soluble proteins. To remove the lipophilic yolk components from the crude IgY extract, lipid aggregates large enough to be removed by a conventional centrifugation or filtration, are formed using various precipitants (e.g. polyethylene glycol, dextran sulphate, alginate, caprylic acid, organic solvents), by a simple dilution of the yolk, or by a freezing-thawing of the diluted yolk. Alternatively, some methods of a lower throughput, such as ultracentrifugation, hydrophobic or affinity chromatography, are employed for the first purification stage. serum to egg yolk of their eggs, where it serves as a means of passively protecting the developing chicks an average egg may contain 100~150 mg of yolk immunoglobulin's (IgY), and substantial amounts of specific antibodies may be collected and purified from the eggs of immunized hens. The availability of large amounts of relatively inexpensive IgY from egg yolks makes it feasible to use these antibodies for passive immunization(Journal et al., 2016, Chalghoumi *et al.*,2009, Hodek *et al*.,2013,Kumaran *et al.*,2016).

2.6 Diagnosis of Foot-And-Mouth Disease

Typical cases of FMD are characterized by the formation of vesicles and epithelial erosions of the snout, tongue, hard and soft palate, coronary band, and feet. FMD cannot be distinguished clinically from other vesicular diseases, such as swine vesicular disease (SVD) and vesicular stomatitis (VS). Consequently, laboratory-based tests (virus isolation or demonstration of FMDV antigen or nucleic acid) are required for differential diagnosis (Oem *et al*.,2009). Laboratory diagnosis of FMD can be performed either by detecting the virus or any of its components or by demonstrating the presence of virus-specific antibodies in samples of tissue or fluid (Rios *et al*., 2018).In acutely infected animals, FMDV, its antigens or nucleic acids can be found in a variety of samples including vesicular fluid, epithelial tissue, nasal and oral secretions, esophageal -pharyngeal fluids, blood and milk, and in tissue samples such as myocardium collected at necropsy (Aftosa *et al.*, 2015).

2.6.1 Antigen detection tests

Isolation and propagation of FMDV

Primary isolation of FMD virus is carried out on Baby Hamster Kidney-21 (BHK-21) cells(Ibrahim *etal*.. 2015). *S*erotypes are the primary bovine thyroid, but they are difficult and exclusive and usually lose its susceptibility to FMDV after numerous passages. Primary lamb kidney (LK) cells are very sensitive to FMDV and vary from primary bovine thyroid (BTY) cells in preserving of their sensitivity to FMDV infection after cryopreservation. Cell lines like baby hamster kidney (BHK-21) is much easier to preserve, but are less susceptible to specific animal-derived FMDV (Mahmoud *et al*., 2019).

Propagation of FMDV in BHK-21 cell was confirmed by appearance of CPE comprising rounding and flattening of the cells, breaking down of the intracellular bridges and finally cell death(Mahmud *et al.*, 2018).To achieve adaption of the virus to the production cell line, the virus is first passaged in BHK21 adherent cells until a rapid cytopathogenic effect develops, and then further propagated and expanded in stationary or roller systems(Dill *et al.*, 2018). Tissue culture Infection Dose50 /ml (TCID50 /ml) refer to virus particles per ml which can produce CPE at 50 percent inoculated cells (Mahmud *et al.*, 2018).Viral titers were estimated by endpoint titration with the Spearman-Kärber method and expressed as 50% tissue culture infectious dose (TCID50) per milliliter. Titrations for virus grown on all cell lines were performed on the adherent BHK164 to avoid biasing the results by titrating BHK179-passaged virus on BHK179 cells (Dill *et al*., 2018). The following figure (B) shows the CPE.

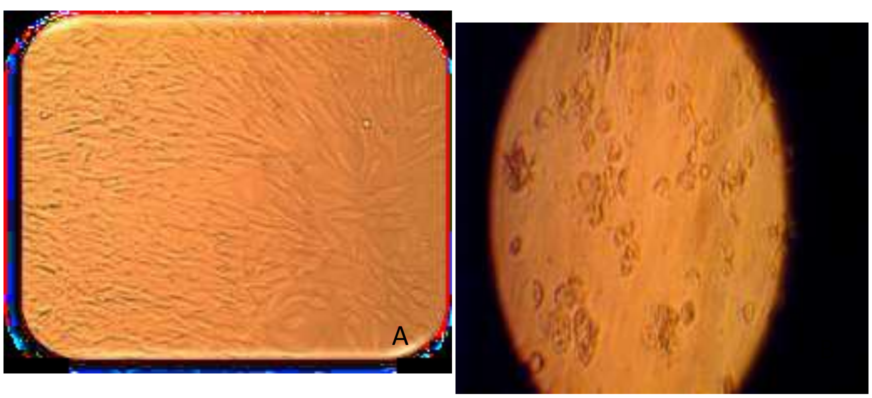

Figure 5: Illustrates the rounding and lysis (CPE) of BHK-21 cells inoculated with vesicular fluid and epithelial tissue samples (A), compared with negative control (B) (Rady et al., 2014).

Sandwich ELISA

Antigen capture sandwich ELISA for virus serotyping is highly sensitive method of virus detection and sero-typing in clinical materials. In this test plates are coated with serotype specific rabbit polyclonal sera and viruses present in processed clinical samples are allowed to bind to capturing antibodies. Bound viruses are detected by serotype specific tracing antibody which increases the specificity of the test. Reaction is developed by tracing antibody specific conjugated antibody and substrate solution(FAO Terrestorial manual,2011). ELISA typing is a qualitative assay for the identification of viral serotype by using indirect double antibody sandwich ELISA. Samples for this assay are suspension of tissue extracts and vesicular fluid.(Megersa *et al.*, 2012).

FMD antigen detection ELISA is shown to be rapid and simpler to perform. The assay is based on the detection of FMDV structural proteins and utilizes the serotype specific polyclonal antibodies generated in guinea pig and rabbits. This antigen-capture sandwich ELISA has 100% specificity for heterologous FMDV and 80% sensitivity for detection of complete virus particles in clinical samples. The assay is easy to perform at regional FMD diagnostic laboratories and large number of samples can be processed without risk of laboratory cross contamination. As the assay specifically detects the intact virion particles in clinical materials in a serotype specific manner, the lower sensitivity could be attributed to the improper storage and transportation of samples that leads breakdown of the virus particles (Sharma *et al.*,2015).

Reverse-transcription PCR (RT-PCR)

The reverse-transcription PCR (RT-PCR) can be used to amplify the genome fragment of FMD virus in diagnostic material. Specific primers have been designed between each of the seven serotypes (Admassu et al. 2015). The use of conventional RT-PCR is an effective confirmatory diagnostic procedure in serotyping of FMDV isolates using serotype-specific primers (Rady *et al*.,2014). A number of reverse transcription polymerase chain reaction (RT-PCR) assays have been developed as an alternative to viral culture but these assays require other laboratory techniques to detect the amplified product, which is associated with considerable hands-on time, poses a serious hazard for amplification product carryover, and limits the number of specimens that can be processed simultaneously. Recently, the development of a real-time reverse transcription polymerase chain reaction (rRT-PCR) procedure has provided an additional tool which can be used for FMD diagnosis (El-shehawy *et al*.,2012).

The application of reverse transcription quantitative PCR (RT-qPCR) provides a high sensitivity reduced risk of cross-contamination and considers as a valuable tool for the detection of viruses. The time required for the common PCR protocols is about 90-120 min, this time could be reduced using recently developed high-speed (PCR) assays for detection of e.g., Influenza, adenoviruses or classical swine fever virus(Abd *et al.*,2015).Development of real-time PCR has facilitated rapid quantitative analysis with reduced amount of post-PCR processing steps. Monitoring PCR product formation in real time can be achieved through the use of dual-labeled hydrolysis (Taq Man) probes consisting of complementary sequences within the target(Subramanian e*t al.*, 2012).The quantification arises by measuring the amount of amplified product at each stage during the PCR cycles. Quantification of amplified product is obtained using fluorescent SYBR green. SYBR green is a dye bind to double stranded DNA. The intensity of fluorescent emissions increase as more double stranded amplicon is produced with the dye signal increase (El-shehawy *et al.*,2012).

The universal reverse primer (BES-VP1R) was designed from the conserved 2B region based on the alignment of VP1 genomic sequences of serotype selected from the Gen-Bank nucleotide database. serotype-specific forward primers were designed from the hyper variable regions of the capsid coding gene (VP1/1D) following the alignments of VP1 (1D) gene sequences of serotypes selected from the Gen Bank nucleotide database and conserved sequences unique to each serotype were identified and used for primer design (Sareyyüpoğlu *et al.*, 2017).

2.6.2 Serological tests

Serological tests are widely used to access historical exposure of animals to foot-and-mouth disease virus (FMDV) or FMDV vaccines based on the immune status of animals(King *et al.*, 2015). Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs). The SP tests are serotype-specific and detect antibodies elicited by vaccination and infection; examples are the virus neutralization test (VNT), the solid-phase competition ELISA and the liquid phase blocking ELISA. These tests are serotype-specific and are highly sensitive, providing that the virus or antigen used in the test is closely matched to the strain circulating in the field(Oie *et* *al*.,2009). Several assays for detection of antibodies to FMDV non-structural proteins have been developed. Most of the assays developed are indirect ELISA's using species specific conjugates, which makes simultaneous examination of sera from different species difficult. Previously, we developed a blocking ELISA, which was species independent. This used baculovirus expressed FMDV non-structural proteins as antigen and polyclonal antibodies produced in guinea pigs as capture and detector antibodies(Sørensen *et al*.,2005).

Serological methods which detect antibodies to FMDV SP such as the liquid phase blocking ELISA, the solid phase competition ELISA or the virus neutralization test cannot distinguish between infection and vaccination with conventional vaccines. However, ELISAs that measure antibodies to different NSP (3ABC, 3AB, 3A, 3B, 2A, 2B and 2C) can be used as marker tests to detect infection in conventionally vaccinated animals. Improved FMD marker vaccines and tests may be available in the future. For example, the experimental adenovirus vectored vaccines described above express recombinant viral capsids that are devoid of NSP 3D, which is a protein that elicits a strong antibody response but cannot be purified away from conventional vaccines. This would enable use of a companion marker test for infection based on detection of anti-3D antibody. Testing of saliva for FMDV-specific IgA is also a promising tool for detection of infection in animals given conventional vaccine by the parenteral route (Vannier *et al*.,2007). However, there is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti-NSP tests, causing false-negative results (Oie., 2018)

Enzyme-linked immunosorbent assay (ELISA)

Currently, immuno-enzyme assays, in the form of commercial enzyme-linked immunosorbent assay (ELISA) kits, are the most common serological tests for the diagnosis of diseases. In all these kits, detector secondary antibodies labeled with an enzyme used to recognize chicken immunoglobulin (Ig) G molecules(Reza *et al.*,2018). Enzyme-linked immunosorbent assay (ELISA) is a simple and rapid method based on antigen and antibody recognition and is highly specific for particular residues. There are various forms of ELISA methods. Direct ELISA is the simplest one, and other methods are indirect ELISA, sandwich ELISA, competitive ELISA, and direct competitive ELISA (Tian *et al*.,2018).

ELISA is a powerful method for estimating Nano gram to picogram quantities of antigen or antibody in a solution; such as serum, urine and culture supernatant. ELISA is used in diagnosis and testing of disease, which detects the presence of either antibody or an antigen in the sample. The key feature of ELISA is the use of immune detection to identify a specific protein, for example a protein marker for a disease. The proteins are immobilized in a protein binding well and non-specific sites are then blocked. The blocking step is used to increase the specificity of the ELISA technique by preventing non-specific interactions. If the wells are not blocked, then the antibodies can stick to non-specific proteins due to their charge. To prevent this, the wells are incubated with a protein mixture and the proteins block the charges that would attract the antibodies. Several blocking agents are used, including dried milk powder, bovine serum albumin and casein; however modern blocking agents use synthetic and/or non-animal proteins to prevent any cross reaction with the animal antibodies (Veerasami *et al*.,2008). For each sample, a net OD was calculated by subtracting the reading of the negative antigen well from the positive-antigen well. For each plate, the net ODs of test samples were then divided by the net OD of the positive control sample on the same plate. Identical aliquots of the same positive control were used for all plates, and results are reported as fractions of the net OD of the positive control (nFPC).To further correct for non-specific reactivity, the nFPC value of the sample taken on the day of first exposure to FMDV (either vaccination or challenge) was subtracted from the nFPC values of all subsequent samples of the same animal (Eschbaumer *et al.*,2016). The 3ABC ® FMDV NS ELISA was used for detection of antibodies directed against the nonstructural protein of FMDV in serum of cattle and buffaloes (Ateya *et al*.,2017).

Viral Neutralization Test

Antibody titers was expressed in the reciprocal of the highest dilution that completely inhibited in 50% of viral CPE per wells (Vaccine *et al.*, 200,Nishimura *et al.*,2002). The antibody response is crucial for preventing many viral infections and may also contribute to resolution of infection. When a vertebrate is infected with a virus, antibodies are produced against many epitopes on multiple virus proteins. A subset of these antibodies can block virus infection by a process that is called *neutralization*. They may interfere with virion binding to receptors, block uptake into cells, prevent un coating of the genomes in endosomes, or cause aggregation of virus particles. Many enveloped viruses are lysed when antiviral antibodies and serum complement disrupt membranes (Rey *et al*.,2017). Serum virus neutralization tests are highly sensitive and specific assays performed in four steps that include (1) serum dilution, (2)viral and antiserum pre incubation, (3) inoculation, and (4) detection (Gauger *et al*.,2014).

The quantitative VN micro test for FMD antibody is performed with IB-RS-2, BHK-21, lamb or pig kidney cells in flat-bottomed tissue-culture grade microtiter plates. The sera are inactivated at 56°C for 30 minutes before testing. The control standard serum is 21-day convalescent or post-vaccination serum. A suitable medium is Eagle's complete medium/LYH (Hank's balanced salt solution with yeast lactalbumin hydrolysate) with hepes buffer and antibiotics (Oie manual,2009).This assay detects antibody that is capable of inhibiting virus replication (or in other words, antibody that can neutralize virus infection). Virus neutralization is a specialized type of immunoassay because it does not detect all antigen–antibody reactions. It only detects antibody that can block virus replication. This is important because related groups of viruses may share common antigens, but only a fraction of these antigens are targets of neutralizing antibody. A virus serotype is usually based on virus neutralization (although this is not always specified) (Payne *et al*.,2017).

Till date five neutralizing antigenic sites (1–5) have been reported for FMDV through monoclonal antibody (mab) neutralization resistant (mar) mutant studies. The βG-βH loop and carboxyl terminus of VP1 contribute to antigenic site 1 with critical residues at positions 144, 148, 154, and 208. Amino acid residues at position 70–73, 75, 77, and 131 of VP2 contribute to antigenic site 2. In addition, amino acid residues at position 79 and 134 have been reported to influence binding of site 2 mabs. Antigenic site 3 is formed in part by residue 43 and 44 of the βB-βC loop of VP1 whereas amino acid residues at position 56 and 58 of VP3 have been reported to be critical for antigenic site 4. Antigenic site 5, characterized by an amino acid at position 149 of VP1, is probably formed by interaction of the VP1 loop region with other surface-oriented amino acids. . Recently attempts have been made to predict epitopes using capsid sequence data and 3-D structure of FMD, and these epitopes can be tested through mutational analysis by reverse genetics (Mahapatra *et al*., 2018).

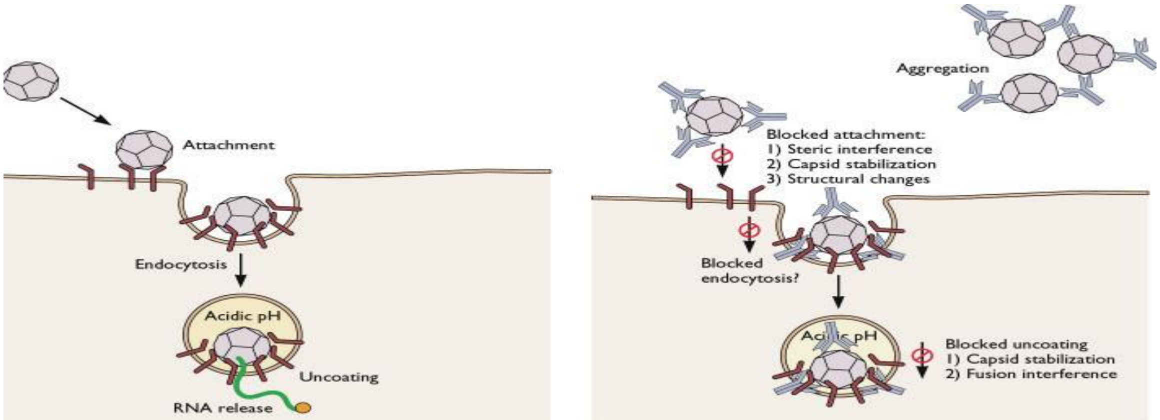

Figure 6*: Mechanism of viral neutralization (source:Mahapatra* et al.*, 2018)*

2.7 Epidemiology of FMDV in Ethiopia

In Ethiopia, FMD is endemic and a notifiable disease; the national animal health regulatory directorate sends monthly and annually official reports to OIE. The disease is widely prevalent and previously used to occur frequently in the pastoral herds of the marginal lowland areas of the country. However, this trend has been changed and currently the disease is also frequently noted in the highlands of the country (Tadesse *et al*., 2018).Among the seven serotypes of FMD viruses, five of them (O, A, C, SAT1 and SAT2) have been diagnosed in Ethiopia at different times. The most dominant serotype is O, accounting for 72% of the investigated outbreaks occurring in the country, followed by A (19.5%). Serotype C has not been reported in Ethiopia since 1983. Seven top types are identified within the four serotypes of FMD viruses that have been reported in recent times in Ethiopia (Jemberu *et al.*,2016).

FMDV was first recorded in Ethiopia in all production systems since 1957. A large number of outbreaks were reported every year. Based on data over the years 2007–2012, the annual district-level incidence of FMD outbreaks was estimated at 0.24, 0.39 and 0.85 per district year in the CLM, pastoral and market-oriented systems, that are caused by serotypes O, A, SAT 2 and SAT 1 (Tadesse *et al.*, 2019).According to (Ayelete *et al*, 2012), the highest sero-positivity was obtained from the Eastern zone of the Tigray region, with 41.5%; followed by the Guji zone of Oromia and the Yeka district of Addis Ababa, with 32.7% and 30%, respectively. The estimated economic losses of FMD outbreak in cattle, arising from milk loss, mortality, and draft power loss, average US$76 per affected herd, US$9.8 per head in crop-livestock mixed system and US$174 per affected herd and US$5.3 per head in the pastoral system. In another study, the overall short-term farm level direct loss due to FMD outbreak in an urban dairy farm was estimated at ETB45, 131, equivalent to €1962. The overall herd- and animal-level prevalence is 57.6% and 11.9%, respectively Data on national level economic impact of FMD is lacking (Thapa *et al*.,2016).

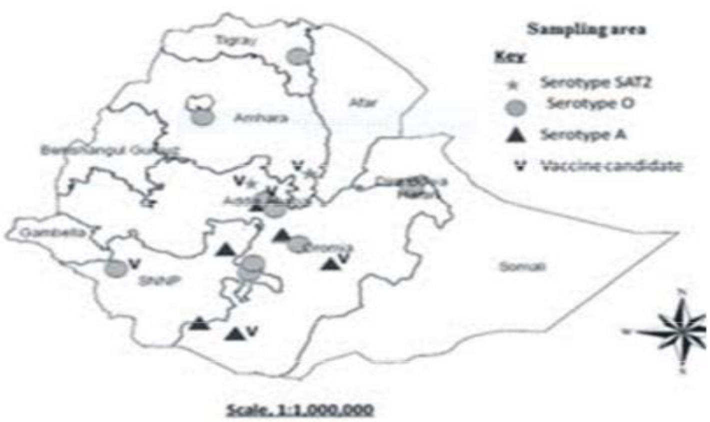

Figure 7: Map of Ethiopia showing the distribution of FMD virus serotype O and A isolated in Ethiopia (Source: Ayelet *et al.*, 2009).

2.8 Morbidity and Mortality

FMD is characterized by high morbidity and low mortality rate (Ibrahim *et al*.,2015).Variation in the morbidity rate occurs and may depend on species, age, sex as well as the status of the immunity. Self-recovery in the animals is the result of immunity against the infecting serotype of the virus. Mostly the disease occurs due to one type of virus and development of immunity also remains confined against specific serotype, thus no immunity develops to other serotypes, a reason behind occurrence of the disease in the endemic areas. The presence of a single serotype in an area or population lead to clinical disease that may be in mild form and mainly infects young animals because of loss of protection from antibodies from the dam (Chakraborty *et al*.,, 2014).

2.9 Economic Impact and Global Distribution of FMD

The national and international trading of a country strengthens its backbone. FMD hinders the trading of milk, meat, animals and other agricultural products. The losses of FMD in terms of production, mortality, and import-export are too much to economically weaken any country. The direct and indirect losses associated with FMD are in terms of mortality, morbidity and milk production, growth rate and abortion (Naveed *et al*.,2018). Recommendations set by OIE policy currently suggest quarantine and slaughter in response to an FMD outbreak in an FMD free country. This involves 'stamping out' of all animals infected and those in contact or at risk of infection (i.e. herds at neighboring premises). Although the mortality of FMD is limited the disease has numerous more constant affects. Knight and Rushton highlight that as well as the immediate loss of production, the long-term productivity can be affected by changes in the structure of the herd due to lower fertility. The cost of managing disease through diagnostics, vaccination and movement control is high but without those controls access to the international markets cannot be achieved. Vaccination and production losses caused by FMD are estimated to cost 5 billion USD per annum globally (Logan *et al*., 2017).

2.10 Prevention and control of FMDV

Because FMD is a viral disease, there is no treatment for the sick animal, and as a notifiable disease it should be eradicated (*Kuva et al*.,2011). The disease is controlled through mass vaccination, selective culling and implementing strict bio-security measures (Muhammad *et al*.,2011). To control FMD effectively, there is need of good infrastructure, trained veterinary staff, well equipped laboratories, good governance, rapid and accurate diagnostics, rapid response measures, continuous monitoring and surveillance, and compulsory vaccination. There is requirement of initial implementation of test and slaughter policy of all infected as well as susceptible animals (at close proximity) for controlling FMD in a disease-free country with movement restriction of susceptible animals, disinfecting infective premises and intensified surveillance to prevent further spread. Restriction over the import of suspected livestock or animal products including fresh meat from countries where FMD prevails is essential. For the development of an efficacious strategy of vaccination it is important to understand the disease dynamics. It indicates the suitable time points to administer vaccine (Rodriguez *et al*.,2009 , Chakraborty *et al*., 2014).

There are presently only 3 interventions practiced in sub-Saharan Africa against FMD: Vaccination of domestic animal populations (usually only cattle) in which infection with one or more (usually several) serotypes is endemic or where the cattle population is at risk of infection from sympatric or nearby wildlife populations; Separation of infected or high-risk populations of wildlife and/or domestic livestock from uninfected populations; enabled through fencing systems and Careful management of the movement of animals and animal products between localities of different FMD-status or FMD-risk; usually effected through permit systems operated by the official veterinary service of the country concerned (Thomson *et al*.,2011).

The Ethiopian government is keen in to launching an official control program against FMD to reduce production losses and to improve the export trade of animals and animal products. Successful livestock disease control programs, for example through mass vaccination, depend not only on technical and economic feasibilities, but also on the motivation of the farming community to fully participate in the implementation of the control program. Farmers' motivation to implement a specific disease control measure is largely driven by their perceptions of the disease's risk and the effectiveness of available control measures (Wudu *et al*.,2015).

2.10.1 Vaccines and the framework of eradication and control

Control of FMD through effective vaccination of susceptible animals is considered to be the corner stone to eliminate the disease in endemic areas, but it is considered very difficult as the FMDV is an airborne transmitted virus, contagious nature of the disease (Ibrahim *et al*.,2015).Vaccination is of great importance in the protection of livestock in areas where FMD is endemic and continues to be used in conjunction with slaughter in the control of outbreaks in both FMD-free and FMD-endemic areas. Vaccination acts both to protect the individual animal and to provide herd immunity, by reducing the availability of susceptible animals. The FMD vaccines that are currently used worldwide only consist of inactivated vaccines without introducing live vaccines. Inactivated FMD vaccines are commonly produced with as gel- or oil-adjuvant depending on the serotype (Shawky *et al*.,2016). Semi-annual mass vaccination is practiced in endemic countries and ring vaccination around outbreak areas has been conducted in non-endemic countries to complement the depopulation strategy (Jouneau *et al.*, 2020). All foot and mouth disease virus vaccines are based on cell culture-derived preparations of inactivated whole virus. FMD vaccines may be monovalent or polyvalent. The monovalent vaccines using a field derived or outbreak strain. Foot and mouth disease virus vaccines may provide protection within 4-5 days after vaccination. It depends on the vaccine and on the severity of challenge. Vaccination initiates a short period of immunity, requiring revaccination at intervals of every six to twelve months and on occasion more often for protection against heavy challenge (Ateya *et al.*,2017).

2.10.2 Differentiating infected from vaccinated animals (DIVA)

Antibodies against NS proteins is the markers for differentiation of infected animals from vaccinated animals (Bruderer *et al.*,2004). Inactivated vaccine preparations produced from virus-infected cultures contain only low levels of virus infection associated (VIA) antigen. Detection of anti-VIA antibody is used to discriminate between antibody elaborated by vaccination and by infection (Donnell. *et al* .,1996). Effective control strategies can be achieved by diagnostically differentiation of infected from vaccinated animals (Srisombundit *et al*.,2013). FMDV vaccines contain only structural protein (SP) of viral capsid components from which pathogen expressing viral non-structural proteins (NSP) may be removed artificially and elicit a corresponding immune response by host after vaccination. In contrast, during natural infection with FMDV, both the SP and NSP of the virus are expressed and induce a corresponding immune response that can be detected from serum after exposure by serological tests (King *et al*.,2015). The differentiation of infected from vaccinated animal is carried out by Testing for antibody to NSP of FMDV in infected animals. Currently. blocking (antigen-capture) ELISA is suitable for detection of antibody to FMD NSP with its high sensitivity and specificity (Chen *et al*.,2011).

2.10.3 Vaccine Matching

The application of NSP tests after vaccination of animals is dependent on the use of purified, inactivated vaccine that is free (as much as is possible) of NSPs. Differentiation of infected animals from vaccinated animals (DIVA) is essential for proper eradication of FMD by vaccination and the development of carrier animals due to vaccination. Antibody response against FMD viral non-structural proteins has been widely used for this purpose (Ateya *et al.*,2017). Serological test methods have been used to quantify antigenic differences between FMDV structural protein antibodies and thereby to estimate vaccine matching between a vaccine strain and a field isolate. Antigenic analysis of field isolates in relation to vaccine strains, based on virus neutralization tests (VNTs), plays a significant role in evaluating the suitability of existing vaccine strains, although significant variation has been reported with VNTs (Tekleghiorghis *et al.*,2014).

The neutralization titers are used to calculate r1 -values to determine antigenic relationships.95 However, interpretation of the test is plagued by limitations, including the uncertainty as to how well the in vitro matching data actually correlates to in vivo cross-protection, the impact of vaccine potency on protection, and the availability of reference reagents. Furthermore, the use of r1 -values to estimate cross-protection relies on having sufficient repeated measures to overcome the inherent variability of the neutralization titers. A novel way to quantify and visualize antigenic relationships is antigenic cartography.97,98 However, the combination of genetic sequencing and antigenic profiling of the outbreak virus are still useful methods to identify newly emerging or re-emerging virus strains and whether available vaccine strains are likely to provide protection against the outbreak virus or not (Rweyemamu *et al*.,2014).

2. MATERIALS AND METHODS

2.7. Study Design

An experimental study design on FMDV (O, A and SAT-2) polyclonal antibody production trials by using layers hens was conducted from January, 2020 up to August, 2020 G.C. before immunization of the chicken, three field isolates (01940/NAHDIC/O, 01940/NAHDIC/A and01940/NAHDIC/SAT-2) were propagated and adapted on BHK-21. Then titration of each serotype was determined according to the Spearmen- Karbler formula. For each serotype eight 24 weeks layer hens were assigned and Immunized with 50:50 FMD virus (serotype A, O and SAT 2) to oil adjuvants. The immunized chicken was managed, handled and followed for clinical change in wire mesh cage separately. After two weeks of boosting the hens, the serum and eggs were collected at 14, 21 and 28 post immunization days. The blood was collected by plane test tube and transported by ice box to the laboratory. after collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature for 30 minutes and Remove the remained clot by centrifuging at 1,000x g for 10 minutes in a refrigerated centrifuge; immediately transfer the serum to the 1.5 ml cryovial tube and stored in -20 "C until used. The egg of each groups was collected and coded by the code of chicken laid it and stored -20 "C until used. The IgY was extracted and purified with Poly ethylene glycol (PEG-600mg) precipitation method from the egg yolk. The serum and extracted egg-yolk IgY were pooled into four samples for each serotype. Antibody detection was conducted by Prio-CHECK® FMDV Non-structural protein kit (© prionics AG, version 1.0_e, product No.: 7810770) and sample showing inhibition Percent's (%) were subjected to Viral neutralization tests (VNT) for invitro challenges.

2.8. Study Population and Sample Size Determination

The study population constituted of 26-layer hens were purchased from local farming enterprise. The obtained chickens were managed under intensive farming system. Purposive sampling methods was conducted to determine the sample size by considering the appropriateness, cost, handling and management of chickens and Laboratory processing of samples. Accordingly, 24 chickens were selected for treatment groups and 2 for control group. From 26 chickens, to each serotype, eight-layer hens were assigned randomly to three FMDV serotypes.

2.9. Study methodology

2.9.1. BHK monolayer cell cultures (BHK-21) preparation

BHK monolayer cell cultures (BHK-21) (AU/PANVAC) passage 32 was serially passaged, maintained and cultivated in Differential Minimum Essential Medium (DMEM) supplemented with Hanks' and Earle's salts (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) during maintenance and passaging and with 5% FBS during infection experiments and 2 mM L-glutamine. All cell media was supplemented with 100U/ml penicillin and 100µg/ml streptomycin (Shimmon *et al*.,2018). Briefly, First the passage 32 BHK-21 (AU/PANVAC) cells were taken from Liquid nitrogen and thawed in water bath, transfer to 15ml falcon tube and centrifuge at 10,000 rpm for 20 minutes with 5 ml complete media, the pellet was washed with PBS. After adding 3 ml of complete media it was vortexed and transferred to one 25cm^2 polystyrene TPP® tissue culture flasks (Sigma-Aldrich, Europe) and incubated at 37°C and 5% CO_2 for 60 minutes for adsorption of the virus to cells and then 5 ml of maintenance media or growth media (2% minimum effective media) was added and incubated at 37°C and 5% CO_2 in a humidified incubator for 24-72 hrs., the growth media containing 10% fetal calf serum was removed from the 25 cm^2 polystyrene TPP® tissue culture flask (Sigma-Aldrich, Europe). Then the monolayer cells were washed with sterile 1X PBS for 3 times. Trypsin (0.5 milliliter) was added into the flask and gentle tapping to detach the cells. The flask was left in the incubator for 5 min and observed under microscope. 5 mL of media containing cell was poured into flask. Again, the cells were sub cultured as passage 34 in three 25cm^2 polystyrene TPP® tissue culture flasks (Sigma-Aldrich, Europe) culturing flask for three serotypes (O, A and SAT-2) at 37°C and 5% CO2 in a humidified incubator for 24-72 hrs. Until they were confluent to 90% or above.

2.9.2. Adaptation of FMDV in BHK-21 cell culture

Foot and mouth disease virus serotypes (A, O and SAT-2) with high CPE were selected for this study. The viruses propagated in BHK-21 (AU/PANVAC) monolayer cells for adaptation and harvesting to enough amounts for the study. The viruses were inoculated into 34^{th} passage of BHK-21 cell monolayer that had more than 90 percent cell confluence in three $25cm^2$ polystyrene TPP® tissue culture flasks (Sigma-Aldrich, Europe). The growth media from the flask containing BHK-21 cell was removed and then the monolayer cells were washed with 1X sterile PBS for 3 times. One milliliter (1mL) of virus inoculum was added to the 25 cm^2 polystyrene TPP® tissue culture flasks (Sigma-Aldrich, Europe). The inoculums were spread over the monolayer cell by tilting for about 45-60 min for the establishment of better interaction in incubator. Then 7 mL maintenance media was added to flask and returned to the incubator at 37°C and 5% CO_2 in a humidified incubator for 1-2 days. The cells were examined twice daily under inverted microscope until showing characteristic cytopathic effect (CPE).

FMDV purification

The cell culture supernatant was harvested by repeated freezing for 20 minutes and thawing for 3 times and the cell suspension was clarified by centrifugation at 5,000 rpm for 15 min at 4°C (Hassen *et al*.,2016, Mahmud *et al.*,2018). These viruses were used for immunization of experimental hens and viral neutralization test.

Virus infectivity and antigenicity

According to, Fawzy *et al* (2015), the titer of the three FMDV serotypes were expressed in log10TCID50.Virus titers in the samples were determined by inoculation of cell culture highly susceptible to FMDV (BHK. -21) (AU/PANVAC) in 96 well flat bottom cell culture plates. Briefly, first a monolayer flask of already cultured cell was taken from incubator, trypsin was added and again incubated at 37°C and 5% CO2 for 5 minutes for detachment. The detached cells were transferred to 15ml of falcon tube and centrifuged at 10,000 rpm for 10minutes. The supernatant was discarded and pellets were dissolved with 3ml of complete media and transferred to new 15ml falcon tube. Then the cell suspension of 2×10^5 cells per ml of growth media were determined by staining cell suspension (0.3ml of PBS and 0.2ml cell suspension) by 0.5ml of trypan Blue solution. Then tenfold serial dilution of viral suspension were also made with media without serum from10^{-1} to 10^{-5} (150µl viral suspension in 1350µl of media without serum), with multichannel pippete 100µl of cell suspension were dispensed to all wells B2-B11, C2-C11,…G2-G11 (2×10^4), in wells of G row (G2-G2) 100µL of media without serum were dispensed and served as negative controls. Then after different 100µl of viral dilution were dispensed on to wells (B2-B11 to F2-F11) in the way that had ten replicates for each dilution, the periphery wells were filled with 200µl of media (so that to made barrier against desiccation) and finally the plates were incubated at 37°C under 5% of CO_2 plus humidity for 72 hrs. The titers were expressed as 50% tissue culture infectious doses (TCID50) per 100 µL calculated with the Spearman-Kärber method (Dill *et al*.,2017).

t =Xo-d (p -0.5)

t: log of dose having cytopathic effect of 50% (TCID50) per volume of inoculation that is 0.1ml

Xo: log of last dilution giving 100% CPE

d: log of dilution factor (d =1 in present case because the dilution is from 10^{-1} to 10^{-5}

P: the sum of proportion of positive wells beginning at lower dilution showing 100% CPE i.e. at dilution Xo titer of virus per ml is

T = 10^{f-t} TCID50/ml

f: log of multiplying factor

2.9.3. Immunization of chickens

Layer hens were purchased from Local Enterprises farm and assign into four groups: Group 1, Group 2, group3 and group 4 for: FMDV Serotype A, O, SAT 2 and Control group respectively. In each three groups 8 hens were included except for group four which contains 2 hens. After 50% tissue culture infectious doses (TCID50) was calculated, the selected FMDV serotype was emulsified with equal volume of oil adjuvant. According to Gmail (Veerasami *et al*.,2008); for FMDV serotype "O" (5.1×10^4 TCID50/ml), "A " (3.7×10^4 TCID50/ml) and for SAT-2 (2.5×10^5 TCID50/ml) were mixed with 10 ml of oil adjuvants. All groups of hens were immunized at an interval of 2 weeks on day 0 and day 14 (boosted) with 1.9×10^3CFU/ml (8.9×10^2CFU/ml at left side and 8.9×10^2CFU/ml at right side of its breast muscle) for group of chicken assigned for FMDV serotype O, 1.3×10^3CFU/ml (6.5×10^2CFU/ml at left side and 6.5×10^2CFU/ml at right side of its breast muscle) for group of chicken assigned for FMDV serotype A and 8.75×10^3CFU/ml (4.375×10^3CPU/ml at left side and 4.375×10^3CPU/ml at right side of its breast muscle) for group of chicken assigned for FMDV serotype SAT-2 (**Table 1**). The eggs and serums from the four groups were collected at day 14, 21 and 28 after the last immunization and stored at 4^o C. The eggs yolk and serum samples were separately kept at -20^o C until used.

Table 1: Determination of Cytopathic Forming Unit Particle for Chicken Immunization

IDNO	FMDV Serotype	Total Dilution Preparation		Injection Per Chicken	
		TCID50/ml	CFU/ml	TCID50/ml	CFU/ml
01940/N AHDIC	O	5.1×10^4 TCID50/ml	3.57×10^4CPU/ml	2.55×10^3 TCID50/ml	1.9 $\times10^3$PFU/ml
8450/N AHDIC	A	3.7×10^4 TCID50/ml	2.5910^4CPU/ml	1.85×10^3TCID50/ml	1.3×10^3PFU/ml
31056/N AHDIC	SAT-2	2.5×10^5 TCID50/ml	1.7510^5CPU/ml	1.25×10^4 TCID50/ml	8.75 $\times10^3$PFU/ml

2.9.4. IgY extraction and Purification

The method for IgY purification was adapted according to Pauly *et al (*2011)**.** The Extraction of chicken IgY antibodies from immunized chicken egg yolk was carried out by using Polyethylene Glycol (PEG 6000) precipitation method. Briefly, once the eggs were cleaned with 75% alcohol, the egg shell was cracked carefully and the yolk was transferred to a "yolk spoon" in order to remove as much egg white as possible. The egg yolk was transferred to and rolled on a filter paper to remove the remaining egg white. The egg yolk skin membrane was cut with lancet and by using the tea spoon the yolks was poured into a 50 ml tube to measure its volume. Twice the egg yolk volume of PBS (PH=7.4) was added to the yolk tube and mixed by vortexing. Then 3.5% of PEG 600g of the total volume was added to the yolk tube, vortexed and rolled for 10 min by a rolling mixer before the tubes were centrifuged at 10,000 rpm for 20 min at 4 ^{o}C. The supernatant was then poured through a folded filter paper and transferred to another tube before 8.5% PEG

6000 was added in relative to the new volume and then vortexed and centrifuged at 10,000 rpm for 20 min at 4 ^{o}C. The supernatant was discarded and the pellet was dissolved in 1 ml PBS using a glass stick, mixed by vortexing before 9 ml of PBS was added to the tube to reach a final volume of 10 ml. The solutions were then mixed with 12% PEG 6000 and the tube centrifuged at 10,000 rpm for 20 min at 4 ^{o}C. The supernatants were discarded and the pellet was dissolved in 800 µl PBS using a glass stick vortexed and the extracts was transferred to 2ml Eppendorf tubes and stored at -20^{o}C until used.

2.10. Detection of FMDV-Specific Antibody

Antibodies against the capsid proteins are produced in both infected and vaccinated animals (FAO. 2007), therefore, Detection and evaluation of antibody titer against FMDV in hen sera/Egg yolk samples was carried out by the Prio- CHECK® FMDV NSP-ELISA kit (© prionics AG, version 1.0_e, product No.: 7810770). In order to enhance the antibody concentration serum and egg yolk, the sample of serum and egg yolk collected from 8 hens for each three FMDV serotypes were pooled in to four for Prio-CHECK ® FMDV NSP-ELISA kit (© prionics AG, version 1.0_e, product No.: 7810770) test. Briefly, according to Prio-CHECK ® FMDV NS kit (© prionics AG, version 1.0_e, product No.: 7810770), first 80 µl of ELISA buffer was dispensed to all wells and 20µl of negative control to (A1 and B1 wells), 20µl of weak positive control (C1 and D1 wells), 20µl of positive control (E1 and F1 wells) and to the remaining wells 20µl of Serum and egg yolk IgY were dispensed and incubated for 18 hrs at room temperature (22 ±3 ºC). then, after six times washing with 200µl washing buffer, 100µl of diluted conjugate were dispensed to all wells and incubated for 1hr at 22 ±3 ºC, again after washing with 200 µl washing buffer, 100 µl of chromogen (TMB) substrate was dispensed to all wells and incubated for 20 minutes at 22 ±3 ºC, finally the optical density was measured at 450nm after adding stop solution and incubating for 20 minutes at 22 ±3 ºC. After the calculation of the mean OD-value of wells A1 and B1 (negative control = OD_{450} max), the percentage of inhibition (PI) of the control and test antibody (egg yolk IgY and serum) was calculated according to the formula of:

$$PI = 100- [\frac{OD450\ test\ sample}{ODmax}] \times 100$$

PI: Percent of inhibition

OD_{max}: Maximum optical density

Interpretation of PI:

PI ≥ 51: positive

PI = 50: doubtful

PI ≤ 49: Negative

3.3 Virus neutralization test

Virus neutralization test is to detect a specific reaction between antigen and antibody or neutralization of antigen to inhibit the viral infectivity. The 21 samples with titers ≥1% of inhibition in the Prio-CHECK® FMDV NS kit (© prionics AG, version 1.0_e, product No.: 7810770) for antibodies against O, A and SAT- 2 were subjected to VNT test. The protocol was adapted from the procedure described in the OIE terrestrial manual (OIE, 2012). **Briefly**, first antibodies were inactivated at 56 ºC for 30-minute incubation in water bath. Then two-fold serially diluted sera and IgY starting from original dilution up to 10^{-10} in modified Eagle's medium was used, From each dilution, 50 µl serum and IgY samples were added in each wells (duplicated). Next 50µl containing 100 $TCID_{50}$ FMDV (previously titrated) were added to each well and incubated at 37°C in CO_2 incubator for 1hr to allow neutralization, then 50µl of 10^6 cells/ml BHK-21 cells suspension was added to each well and incubated at 37°C of 5% CO2 incubator for 48 hr., the wells were examined microscopically for the presence of CPE daily. After its incubation of 48 hr. the presence of CPE was examined and further stained by methylene blue for 30 min after which excess stain was discarded, finally, the plates were washed with distilled water for 5 times and left for 30 min to dry in the incubator. The neutralization titer in log10 was calculated according to Spearman-Kaerber based on FMDV-induced cytopathogenic effect (CPE).

Calculating TCID50/ml= xk + d [0.5 – (1/n) (r)]

While: xk = dose of the highest dilution.

r = sum of the number of “-" responses.

d = spacing between dilutions.

n = wells per dilution.

3.4 Data Analysis and Management

Data obtained from laboratory tests; virus titration, virus neutralization, and ELISA tests were entered into Microsoft Excel before being analyzed. Descriptive statistics was carried out using Microsoft Excel spreadsheet to summarize the results. The log 10 of VNT were Calculated by Spearman-Kärber method (Dill *et al*.,2017) on Microsoft excel. Analysis of variance (ANOVA) and unpaired t. test were performed to assess the significant difference between IgY and serum induced by the three serotypes of FMDV, between antiviral (IgY and serum) and between three post infection days by using RStudio packages.

4 RESULTS

4.1 The PrioCHECK ® FMDV NSP-ELISA

Among the sixty pooled samples collected from immunized chicken during April, 2020, only in nineteen (32%) sample low antibody against the selected serotype of FMDV were detected by Prio-CHECK® FMDV NS kit. To characterize the polyclonal antibody–binding sites, their reactivity against the homologous FMDV (serotype: A, O and SAT-2) were examined using Prio-CHECK® FMDV NS kit Their group produced polyclonal antibody were failed to react with homologous FMDV (serotype: A, O and SAT-2) to standardized percentage of inhibition, indicating that their binding sites were conformational. At 14-day post immunization, the Prio-CHECK® FMDV NS kit. Antibody detection in both the egg yolk IgY and serum all groups reached (10.6 ± 8.85 and 0, respectively), then began to reduce at 21 at day post immunization to (8.6 ± 6.9 and 4.7 ± 3.4, respectively). Again, it increased at day 28 post immunization to (13.5 ± 9.8 and 6.7 ± 5.9, respectively) (**Figure 8-10**).

4.1.1 Day 14 post immunization

At day 14 of post immunization, the egg yolk IgY antibody mean inhibition percentage (PI) was (10 ± 8.9), while, the serum antibody inhibition percentage was not detectable (**Figure 8**). Also, there were the significant difference in antibodies percent of inhibition between the egg yolk from the different hens within the groups of chickens infected with the same serotypes of FMDV as indicated by error bar (Figure 8). The statistical significancy between egg yolk IgY and serum IgG was significantly different (P-Value = 0.04) as described by paired t-test.

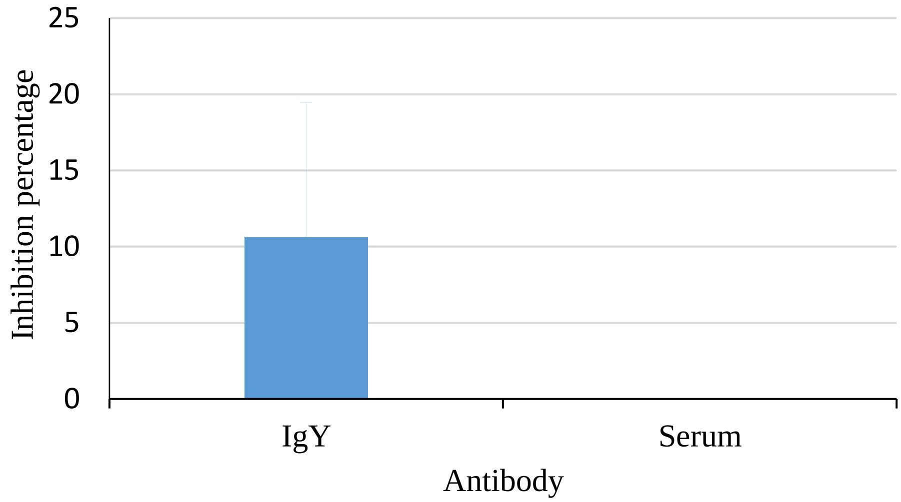

Figure 8: Comparative mean of percentage inhibition (PI) values of egg Yolk IgY and serum at day 14 post immunization of a chicken (n = 5) immunized by three serotypes of foot-and-mouth disease.

4.1.2 Day 21 post immunization

On contrary to day 14, the serum percentage of inhibition increased, while the egg yolk IgY sh owed a decline on day 21. In both the egg yolk IgY and serum there was a higher percentage of inhibition deviation **(Figure 9**) between individual within the groups of chicken immunized wi th FMDV (serotype: A, O and SAT-2). The percentage of inhibition were (8.6 ± 6.9 and 4.7 ± 3 .4) for egg yolk IgY and serum respectively. The difference between egg yolk IgY and serum w ere not statistically significant as analyzed by paired t-test (P-Value > 0.05).

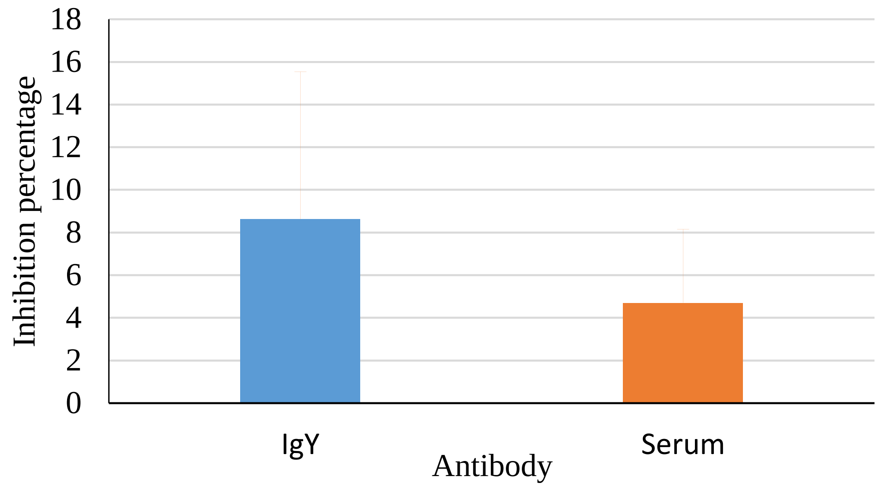

Figure 9: Comparative mean distribution of percentage inhibition (PI) values of IgY and serum at day 21 post immunization of a chicken (n = 6) immunized by foot-and-mouth disease.

4.1.3 Day 28 post immunization

As compared with day 21 post immunization, there was an increase in the percentage of inhibition on both egg yolk IgY and serum (**Figure 10).** Within all groups the individual percentage of inhibition deviation were high as at day 14 and 21 post immunization of the chicken with FMDV (serotype: A, O and SAT-2) (13.46 ± 9.8 and 6.7 ± 5.9) for egg yolk IgY and serum respectively. The difference between egg yolk IgY and serum were not statistically significant as analyzed by unequal variance t-test (P-Value > 0.05).

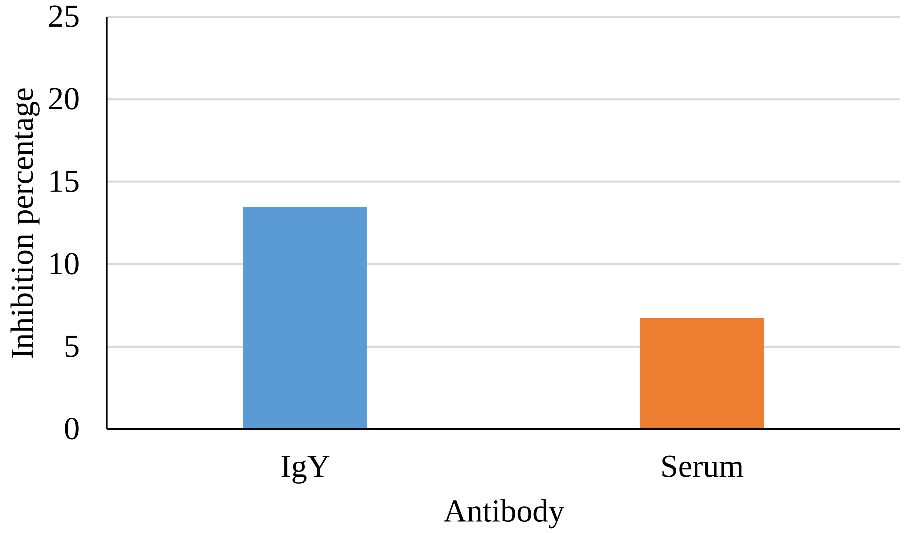

Figure 10: Comparative mean distribution of percentage inhibition (PI) values of IgY and serum at day 28 post immunization of a chicken (n = 8) immunized by foot-and-mouth disease.

4.1.4 Response of Chicken to the different Serotype of FMDV

The detection antibodies of immunized chicken to FMDV (serotype O, A and SAT-2) were differ between the groups. The mean inhibition percentage of serotype A were high as compared with O and SAT-2 serotypes (16.1, 10.24 and 3.15, respectively) (**Figure 11**). The variation between the groups of chicken analyzed by the analysis of variance (ANOVA) tests. The statistical result shown that there was significant difference between the group immunized by three different serotypes (P-value = 0.03). As indicated by error bar, the percentage of inhibition deviation between individual in each groups of chicken immunized by different serotypes were high (16.1 ± 9.3, 10.24 ± 6.9 and 3.15 ± 2.1, respectively).

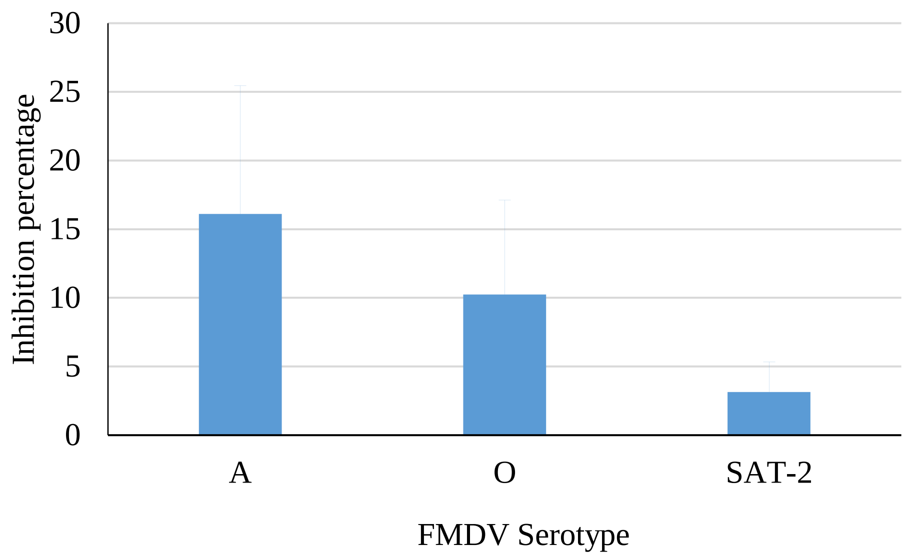

Figure 11: Comparative mean of percentage inhibition (PI) values of IgY and serum between the groups of chicken (n =19) immunized by the serotype of FMDV (A, O and SAT-2).

4.2 Virus Neutralization

Neutralization tests against three FMDV serotypes (A, O and SAT-2) were carried out on each serum and IgY detected by prio-CHECK® FMDV NS kit. Out of sixteen pooled sample detected by Prio-CHECK FMDV NS kit, 19 sample which had shown the low antibody against the selected serotype of FMDV. In contrast to their recognition of antibody-captured virus in the prio-CHECK® FMDV NS kit, all samples were positive for neutralization tests against three FMDV serotypes (A, O and SAT-2). The statistical analysis showed that antibody titer against three FMDV serotypes was significantly different (P-Value < 0.05) (**Figure 15**). In time spans of 2 to 4 weeks, the antibody level was in increased state against the three serotypes from day 14 (**Figure 12**) post immunization to day 21 (**Figure 13**) post immunization, but decreased from day 21 to day 28 (**Figure 14)** post immunizations, but the difference between days post immunizations were not statistically significant (P-value < 0.05). The neutralizing titer of the IgY and the serum was evaluated in the association between the post immunization day, between the serotype and between antibodies (Serum and IgY) by using analysis of variance (ANOVA) and paired t-test analysis. It's statically expressed in significant of P-value. Statistically only the relationship between the serotypes had the significant impacts on the neutralizing antibody titer against the three serotypes of FMDV (P-Value = 0.03).

4.2.1 Day 14 post immunization

During day 14 post immunization the mean of egg Yolk IgY titer against the FMDV (serotype; O, A and SAT-2) was (mean = 1.83), however, all serum collected from the same group of chicken were negative (**Figure 12**). The relationship between the egg yolk IgY and serum analyzed by t-test of equal variance and their relationship was significant (P-Value = 0.03). As described by error bar (**Figure 12**), the deviation between individual grouped as Egg yolk IgY were very low (1.83 ± 0.2).

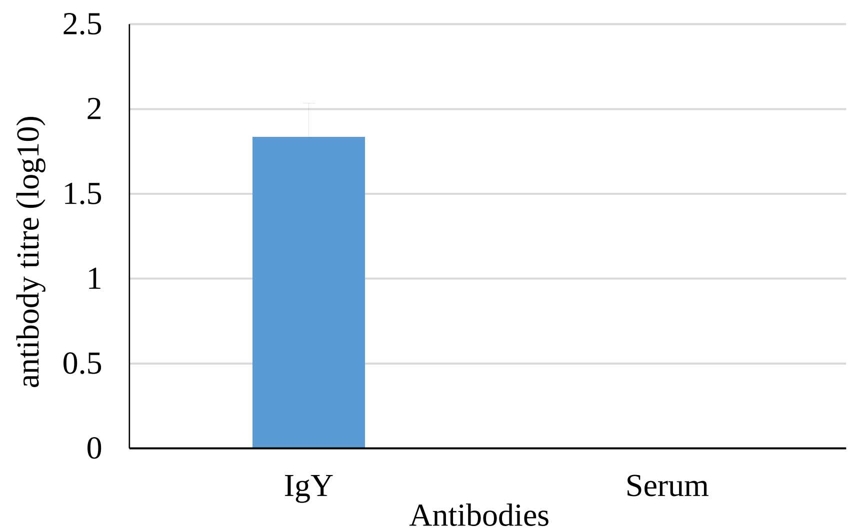

Figure 12: Mean and standard deviation of antibody titer induced by FMDV (serotype: O, A and SAT-2) at day 14 chicken (n = 5) post immunization.

4.2.2 Day 21 post immunization

At day 21 post immunization IgY mean titration increased to (mean = 2.1), while the serum means antibody titration increased from zero at day 14 post immunization to (mean = 1.90) (**Figure 13**). These increase in antibody titer implies that the second injection at day 14 acts as booster of antibody responses by the immunized Chickens. Still day 21 the mean antibody titer in the egg yolk was greater than that of serum collected from layer, however, the difference in antibody titration between egg yolk IgY and serum was not statistically significant (P-Value = 0.43).

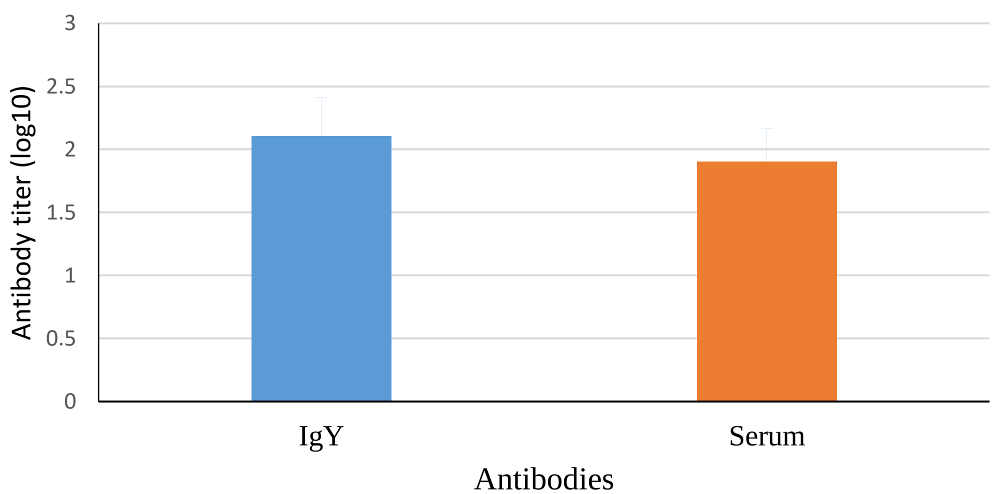

Figure 13: Mean and standard deviation of antibody titer induced by FMDV (serotype: O, A and SAT-2) at day 21 chicken post immunization (n = 6).

4.2.3 Day 28 post immunization

The mean titer values shown below are within each group for the dilution of sera and IgY, which resulted in a reduction in CPE formation when compared with that of day 21 post immunization of chickens (mean= 2.07) (**Figure 14**). The results also showed that IgY mean antibody titer exceeds the mean antibody titer of serum, however, the difference between them were not statistically significant (P-Value > 0.05).

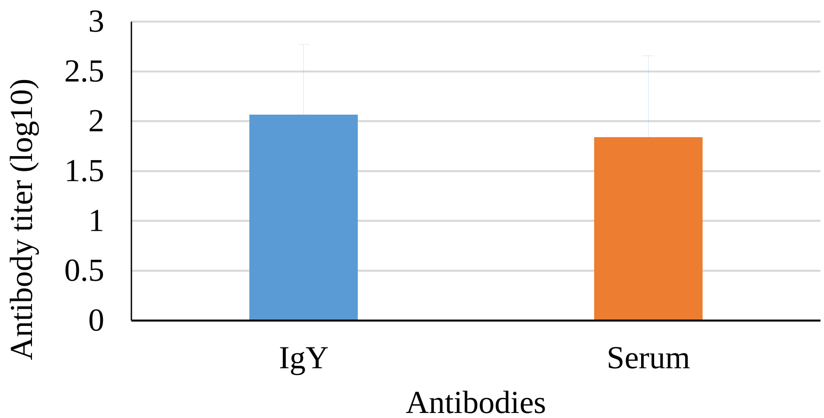

Figure 14: Mean and standard deviation FMDV (serotype: O, A and SAT-2) specific antibodies in sera and Egg Yolk from each chicken group at day 28 post-immunization (n =8).

4.2.4 Response of Chicken to the different Serotype of FMDV

The below figure shown the mean antibody titer induced by serotype A was the highest, while the SAT-2 was induced low (A = 2.25, O =2.07 and SAT-2 = 1.59, for FMDV serotype A, O and SAT-2 respectively) (**Figure 15**). Stereotypically, the neutralizing antibody titer induced in chickens by FMDV (serotype A, O and SAT-2) were statistically different (P-Value = 0.0392) between the groups of immunized chickens.

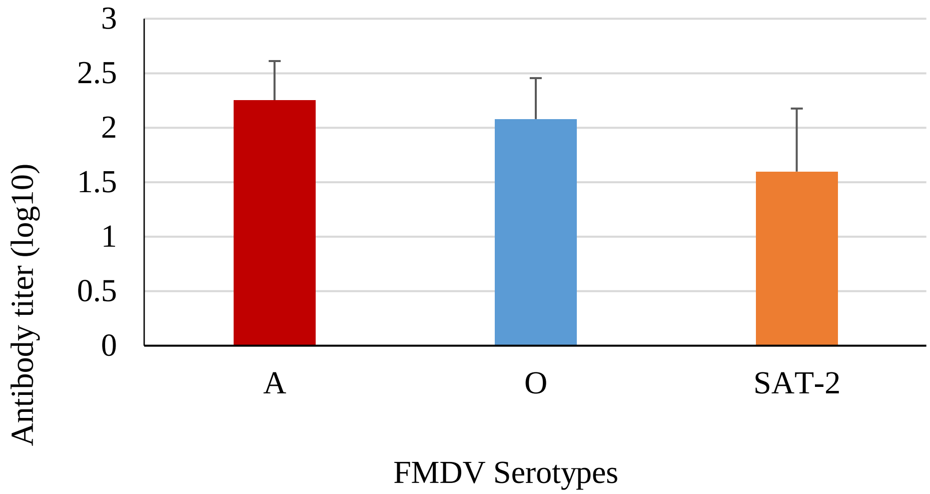

Figure 15: Comparation of total antibody titer induced by immunized chicken (n =21) to FMDV (serotype: A, O and SAT-2).

4.2.5 Mean comparation of Serum and egg Yolk IgY

General mean comparation of Serum and egg Yolk IgY, collected from immunized chicken shown (**Figure 16**). The mean of Egg yolk IgY exceed the that of serum (2 and 1.9, respectively). When it's compared by unequal variance t-test, the difference between the Egg yolk IgY and serum were not statistically significant (p-Value >0.6).

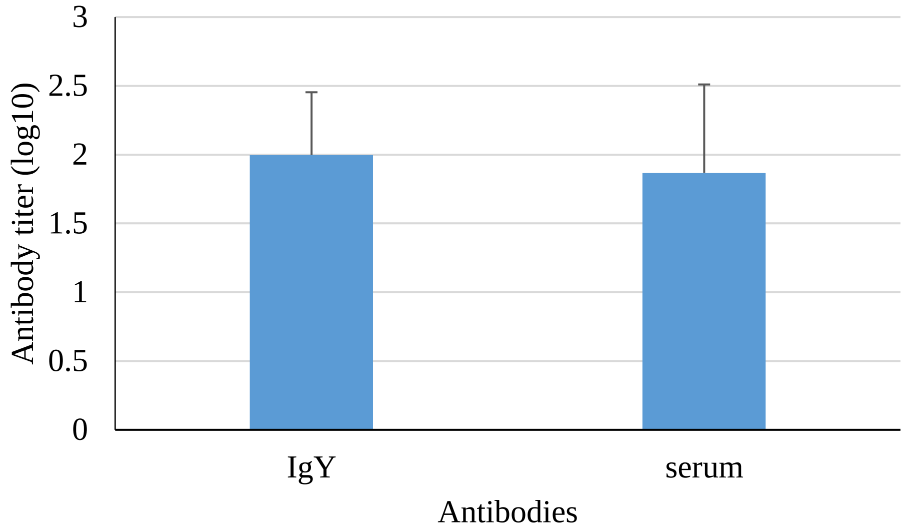

Figure 16: General comparation of Chicken IgY and serum titer (n =21) against the FMDV (serotype: O, A and SAT-2).

5 DISCUSSION

FMDV is an important Livestock disease, which mainly affect the wild and domestic cloven-hoofed animals (Juleff *et al*.,2009). Antibodies play a significant role in the host neutralizing the FMDV infection(Reeve et al. 2016). Antibodies produced by infection or vaccination play an important role in protection of viral infection, in which plasma and memory B-cells keeps its protection ability for secondary exposure to the same infection (Grant *et al*.,2016). Different strains of FMDV vary considerably in pathogenicity, invasiveness, virulence spreading power and prevalence of occult, or in apparent infection (Vallce 1961). Accurate diagnosis and vaccination are significant measure for controlling of the disease. Both diagnosis and vaccination need specific antibody against the targeted viral disease. Therefore, the objectives of the current study were to produce Foot-and-mouth disease (Serotype: O, A and SAT-2) polyclonal antibodies (IgY) by using layer hens. In selecting a laboratory animal to model FMDV immunity, a number of factors must first be considered: animals must be susceptible to infection, support viral replication and the immune response must play an active role in controlling infection. When FMDV antigen infect the host species mutated in order to adapt its self to host cell, the antibody produced will not recognize the epitope of original antigen, as mutations on antigenic sites have been destroy binding with relevant virus-specific antibodies in FMDV (Mahapatra *et al*., 2012)

Normally the FMD virus is characterized by the signs of fever, vesicles in the mouth, feet and udders, loss of production and death in young animals (Lv *et al*.,2009), however the live virus immunized hens did not show the sign of fever, vesicle, loss in egg production and no death of immunized chickens. According to, Skinner., (1954), there was no such systematic disturbance in chicken of all ages seen under this study. The other point to bared in mind was experimentally, other species, including mice, rats, guinea pigs, rabbits, embryonating chicken eggs, and chickens, may be infected, but this often requires artificial transmission of the virus, and infection of these species has not been implicated in significant spread of FMDV

The antibodies detection for both the serum and egg yolk antibodies were carried out by prio-CHECK FMDV NS ELISA kit. The Prio-CHECK® FMDV NS kit detection results were below the standard cut-off values (< 50%) of percentage of inhibition described on prio-CHECK kits (PI > 50 were interpreted as positive). At day 14, 21 and 28 post immunization of chickens the mean percentage of inhibition of Egg yolk IgY ((10 ± 8.9, 8.6 ± 6.9 and 13.46 ± 9.8) respectively. The obtained EISA results were agreed with the results of (Ferris *et al.*,1998, Bruderer *et al.* 2004). According to, Ahmed Kamal et al, (2017), positive sera may not always gives positive reaction with Prio-CHECK ELISA. This heterogeneity in the 3ABC response is likely to reflect differences in the degree of the viral replication and immunological predisposition to elicit anti-3ABC antibodies (Bruderer et al. 2004). The percentage of inhibition of both serum and egg yolk were different in different day post immunization and between the serotypes of FMDV. The difference in different day post immunization were not had the statistical significance (P-value >0.05), but the difference in different FMDV had statistically significant value (P < 0.05). This indicated the time post immunization and FMDV serotypes had the effects on the antibody response by the chickens against the FMDV. According to, Dekker *et al.*(2016), the time for detection of maternally derived antibodies were differed for the different serotypes of FMDV.

Despite to the Prio-CHECK® FMDV NS kit, when measured by standard neutralization test (100 TCID50 virus–serum dilutions), as comparation by the gay post immunization of chickens the FMDV immunized chicken had relatively high neutralizing antibody titers ranging from 1.83 to 2.164 log10 from day 14 to day 28 post immunization, in which the obtained results were meet the interpretation criteria for antibody titer by Pirbright laboratory manual (> 0.3) log10 titration. As indicated on above the mean antibody titer were increased from day 14 to 21 post immunization and decreased from 21 to day 28 post immunization. This result agreed with the result of(Rodriguez *et a*l. 2003, Eschbaumer *et al*. (2016) on animals vaccinated with the commercial oil-based FMD vaccine in which they had proven that Protection did not depend on the reactivity in the peptide ELISA, rather the protection was associated with the presence of neutralizing antibody titers in vaccinated animals (Rodriguez et al. 2003). Also, the mean antibody titer in log10 of the antibody response of the chicken against the FMDV were compared in the study. The result obtained from serotype comparation show that the response of the chicken to the serotype O were high as compared its mean antibody titer against (**Figure 15).** The difference between the thee serotype, A, O and SAT-2. statistically significant (**P-Value = 0.03**) differenced antibody with the mean log10 titer which agreed with the result of Daoud *et al*.,(2013). The mean antibody titer of Egg yolk and serum also shown that the men antibody titer of IgY were exceed that of the serum antibody, but the difference between egg yolk antibody and serum were not statistically significant (**P-Value >0.05).** The obtained result agreed with the result of Ibrahim *et al*.,2017.

In general, in the current study there were poor correlation between the ELISA antibody detection results and virus neutralization results of both egg yolk antibody and serum antibody. This difference in the two tests indicate that the epitopes of original antigen used for immunization of chicken were mutated during its adaptation to the chicken cells. The mutation can cause change the conformation of epitopes structure which impaired the antigen-antibody interaction during the ELISA tests, however for antibody titer requires only the memory of the originally exposed antigen. The difference between the VNT and Prio-CHECK® FMDV NS Kit perceived antigenic evolution of the virus in which the original virus for immunization of chicken may mutated in order to adapt to chicken cells, however, VNT assays implies the heterologous (cross-protection). According to, Yang), poor correlation between ELISA and VNT might be of individual variation after FMDV inoculation, in which VNTs detect neutralizing antibody activity while ELISA may detect different antibody subsets. i.e. The ELISA detect only specific epitope of original antigen to which animals were exposed with its confirmation, while the neutralizing antibody need only memory of original antigen to which animals were exposed. This indicated that the blocking ELISA didn't indicate the antibody titers which are important for post–vaccine monitoring as VNT test. The viral neutralization of antibody against the specific viral pathogen in secondary exposure of host is occurred as signaled by early passive immunity to the primary infection of cattle and other species(Doel , 1996).

according to, Yang *et al*., 2016, NSP ELISAs test cannot be used in testing of vaccine efficacy and effectiveness. Mutation of the virus may alter the conformation of the epitope which resulting in poor reactivity (Mahapatra *et al*.,2010). According to, Reeve *et al*., (2016), individual mutations may have a large effect on virus antigenicity. Site of neutralization may escape the viral mutation and maintained the cross-protection and residual in vitro neutralization by antisera generated against the parental virus. A possible explanation for the poor binding may be other mutations occurred that changed the conformational structure of the mab binding sites, thus affecting the mabs' binding (Yang *et al*..2016). Aspects of the host cell including mutations, reduced expression of viral receptors, obstacles to viral uptake after receptor events and changes in immune response including cellular immunity, humoral immunity, and cytokine response. However, genetic changes of the virus including production of defective interfering particles, acquisition of new epitopes, the occurrence of replication-defective mutations, and the emergence of variants that replicate with new replication intermediates (Han *et al*.,2018). The majority of peptide molecules used for immunization are likely to present conformations that cannot be adopted by the cognate region in the virus, and it is thus not surprising that many of the antibodies that are induced will be unable to recognize the viral epitopes (Benito *et al*.,1998).

6 CONCLUSION AND RECOMMENDATIONS

In the current study, chickens were experimental infected with three serotypes of FMD viruses (O, A and SAT-2) for production of FMDV polyclonal antibody. After infection on day 14, 21 and 28 the sample of egg and serum were collected. Both the serum and the egg yolk precipitate were detected and evaluated for their antibody's titer by prio-CHECK FMDV NS kit and virus neutralization tests. In 35% of collected samples the antibody against the FMDV were detected with low percentage of inhibition (<50%), but the virus neutralization test results of those sample with low antibodies titer showed value comparable to the OIE standard of antibody titers against FMDV. The mean percentage of inhibition and antibody titer against FMDV in egg yolk (IgY) exceed the amount of antibody titer in the serum. This antibody can be used for any immunological tests applied for FMD diagnosis. Moreover, the study finding indicated that chicken egg yolk antibody (IgY) production could be the best alternative to the other mammalian laboratory animals as egg yolk-based antibody production reduce the stress and bleeding during the blood collection, high antibody titer and low cost, easy to manage and handle chickens.

Based on the finding of this study, the following recommendations are forwarded:

- The research institute and diagnostic laboratory should use the chicken as laboratory animal models for antibody production.
- Before immunization of chicken with virus to which it is not susceptible, in vitro adaptation of virus to the chicken origin cell should be done.
- Repeated immunization and monitoring for longtime duration should be done.
- Further research should be done in order to produce a highly purified antibody used for immunological test for FMDV.
- Sequencing analysis of antigen- antibody determinant epitopes should be done in order to validate the discipancy between antibody ELISA and VNT test result.

- For protein (antibody) quantification, SDS-PAGE and western blotting technique should be applied.
- This study should continue in such a way that the antibody produced can be used in house serotyping EISA test as a replacement of commercially available EISA kits.

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APPENDIX

Appendix 1**:** Dilution formation of viruses for injection of chickens

Serotype OF FMDV	Dilution (virus suspension +DMEM)				Mixture of Virus titer and oil adjuvant (1:1)
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
O	1ml+9ml	1ml (10^{-1}) +9ml	1ml (10^{-2}) +9ml		10ml (10^{-3}) + 10ml (oil adjuvant)
A	1ml+9ml	1ml (10^{-1}) +9ml	1ml (10^{-2}) +9ml		10ml (10^{-3}) + 10ml (oil adjuvant)
SAT-2	1ml+9ml	1ml (10^{-1}) +9ml	1ml (10^{-2}) +9ml	1ml (10^{-3}) +9ml	10ml (10^{-4}) + 10ml (oil adjuvant)

Appendix 2: VNT procedures:

1. Add 50µl of medial to all wells for doing serum/IgY dilution, except column first column for original sample
2. Add 50 µl of serum/IgY to the first column and make serial dilution starting with ¼ (transfer 50 µl from A2-A10 through H2-H10).
3. Add 50 µl of already titred viruses from A1-A10 through H1-H10.
4. Use column 12the for controls (standard antiserum of known titre, a cell control, a medium control, and a virus titration used to calculate the actual virus titre used in the test).
5. A volume of 50 µl of 10^6 cells/ml cell suspension is added to each well.
6. Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days.
7. Microscope readings may be feasible after 48.

Appendix 3: VNT test Plate layout

Sample no		1	2	3	4	5	6	7	8	9	10	11	12
		10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**		**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**Cont.**
1	**A**												**IgY**
	B												**IgY**
2	**C**												**Virus**
	D												**Virus**
3	**E**												**Cell**
	F												**Cell**
4	**G**												**Media**
	H												**Media**

-

Appendix 4: IgY-extraction by means of PEG-precipitation\

1) The eggshell is carefully cracked and the yolk is transferred to a "yolk spoon" in order to remove as much egg white as possible.

2) The yolk is transferred to a filter paper and rolled to remove remaining egg white, then the yolk skin is cut with a lancet or a similar instrument (pipette tip). The yolk is poured into a 50 ml tube and the egg volume is registered (V1).

3) Twice the egg yolk volume of PBS is mixed with the yolk (∑V1+V2), thereafter 3.5 % PEG 6000 (in gram, pulverized) of the total volume is added and vortexed, followed by 10 min rolling on a rolling mixer. That step of the extraction procedure separates the suspension in two phases. One phase consists of "yolk solids and fatty substances" (original quotation of Polson et al. 1980) and a watery phase containing IgY and other proteins.

4) The tubes are centrifuged at 4°C for 20 min (10,000 rpm according to 13,000 x g, Heraeus Multifuge 3SR+, fixed angle rotor). The supernatant (V3) is poured through a folded filter and transferred to a new tube.

5) 8.5 % PEG 6000 in gram (calculated according to the new volume) are added to the tube, vortexed and rolled on a rolling mixer as in step 3.

6) . Repeat step 4 with the difference that the supernatant is discarded.

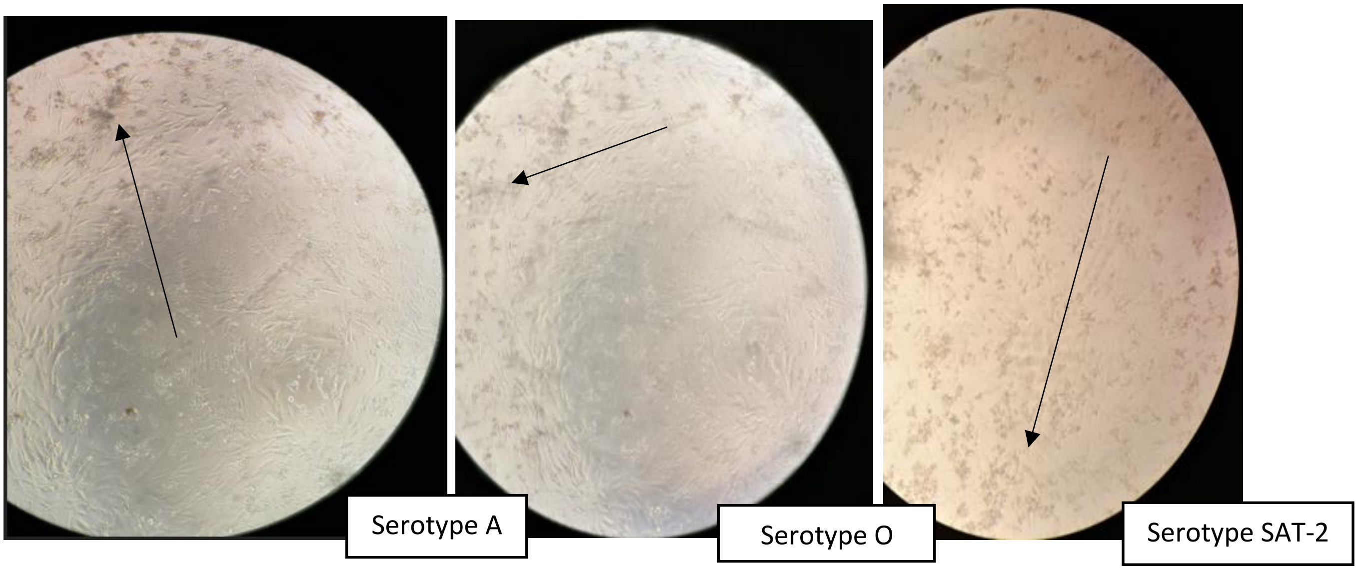
7) The pellet is carefully dissolved in 1 ml PBS by means of a glass stick and the vortexer. PBS is added to a final volume of 10 ml (V4). The solution is mixed with 12 % PEG 6000 (w/v, 1.2 gram) and treated as in step 3 (vortex, rolling mixer

8) Repeat step 6 and dissolve the pellet carefully in 800 µl PBS (glass stick and vortex). Wait for the air bubbles to disappear and then transfer (pipette) the extract to a dialysis capsule. Rinse the tube with 400 µl PBS and add the volume to the dialysis device (V5). (For preparation of dialysis devices and membranes see appendix.)

9) . The extract is dialysed overnight in 0.1 % saline (1,600 ml) and gently stirred by means of a magnetic stirrer. The next morning, the saline is replaced by PBS and dialysed for another three hours.

10) . Thereafter the IgY-extract is pulled from the dialysis capsule by a pipette and transferred to 2ml tubes. The final volume is around 2 ml (V6).

Appendix 5**:** CPE of three FMDV (Serotype A, O and SAT-2)

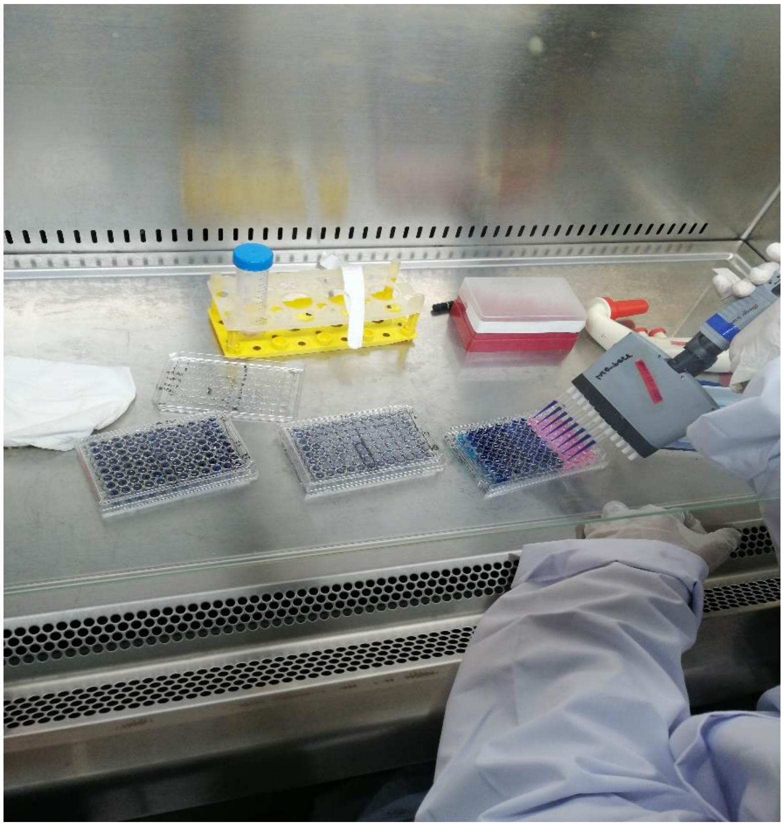


Appendix 6**:** experimentally Immunized chickens

S

Appendix 7: IgY extraction

Appendix 8: Staining of VNT Plates



Appendix 9**:** Microscopic Examination