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Conjugation Of Foot And Mouth Disease IgY In Chicken Egg Yolk With Horse Radish Peroxidase For Typing Of Foot And Mouth Disease Virus

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ABSTRACT

Through the present work, anti-FMD IgY immunoglobulins for serotypes O; A, and SAT2 were prepared in chicken egg yolk through immunization of laying hens with locally prepared trivalent FMD vaccine. The antibody titres against three serotypes (O; A, and SAT2) measured by SNT were 2.58; 2.8 and 2.6 respectively and where 2.81; 2.81 and 2.8 respectively when measured by ELISA. Evaluation of Anti-FMD IgY conjugated with Horse radish peroxidase for detection of FMD virus in 100 tongue epithelium; 50 OP fluid samples; 20 nasal swabs from naturally infected cattle, and 100 tissue culture infected fluids revealed sensitivity of 85% indicating the success of preparation of anti-FMD immunoglobulins conjugated with horse radish peroxidase as local product available on request saving cost and time.

Key words: ELISA, FMD, immunization, PCR, Horse radish peroxidase, serotypes.

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INTRODUCTION

Foot and Mouth disease (FMD) is a highly contagious disease of cattle, sheep, goats and pigs. Foot-and-mouth disease virus (FMDV) is the etiologic agent (a member in genus Aphthovirus in the family Picornaviridae) that can affect cloven-hoofed livestock. The virus exists in the form of seven serologically and genetically distinguishable types (O, A, C, Asia1, SAT1, SAT2, and SAT3) but a large number of subtypes have evolved within each serotype **(Neeta et al., 2011).** The virus spreads by direct contact with infected animals, and indirect contact via contaminated animal products, animate and inanimate objects and by atmospheric dispersal. It causes an acute disease characterized by fever, lameness and vesicular lesions on the feet, tongue, snout and teats, with high morbidity and low mortality **(Depa et al., 2012).**

In Egypt, the disease is enzootic and many outbreaks have been reported since 1950. FMD serotypes SAT2, A and O were last reported in years 1950, 1972 and 2000, respectively **(Aidaros, 2002)**. The type O was the most prevalent since 1960 and onwards **(Farag et al., 2005 and Parida Satya, 2009)**. Serotype A was introduced to Egypt during 2006 through live animal's importation where sever clinical signs were recorded among cattle and buffaloes **(Abd El-Rahman et al., 2006).** In addition, serotype SAT-2 of FMD virus was later introduced to Egypt during 2012 also through live animal's importation **(Shawky et al., 2013)**.

Immunoglobulins IgY are the predominant serum immunoglobulin in birds, reptiles, and amphibians, and transferred from blood (serum) to egg yolk as a maternal immunity to confer passive immunity to their embryos and neonates **(Leslie and Clem, 1969)**. Domestic chickens (Gallus gallus) have been considered as an antibody factory of choice for some decades now and IgY (the primary serum immunoglobulin found in birds and reptiles) has been demonstrated as being efficacious in a range of immunological applications ranging from immunoassays to various proteomics applications **(Karlsson et al., 2004).** Indeed, using chicken as a host for antigen immunization and generation of polyclonal antibodies (IgY) has demonstrated several valuable advantages over existing mammalian systems.

This study was designed as an interesting work aiming to provide a specific kit conjugated with horse radish peroxidase to be available on the time of need to determine and typing FMD virus serotypes.

MATERIALS AND METHODS

Virus serotypes and propagation:

Foot and mouth disease virus serotypes (A IranO5, O Pan Asia and SAT-2/ Egypt 2012) were

supplied by FMD Vaccine Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI). The viruses were propagated twice in primary monolayer of bovine kidney cells then 6 serial passages in BHK21 monolayer cells obtained from Pirbright Surrey, UK according to **Xuan et al., (2011)**. These viruses were used for vaccine preparation; serum neutralization test and antigen preparation for ELISA.

Monovalent FMD vaccine:

Monovalent FMD vaccines (O; A and SAT2) prepared according to **Safy (2011) and Farag et al., (2011)** were supplied by FMD Research Department, VSVRI and used for immunization of experimental chicken.

Immunization of chickens:

Twenty one 6 months old white specific pathogen free laying hens, obtained from Koum Oshem Farm were assign into 3 groups; each group containing 7hens. 3 Freund`s adjuvant monovalent FMDV for each serotypes (A Iran5, O Pan Asia and SAT-2/ Egypt 2012) vaccines were emulsified with an equal volume of Freund's complete adjuvant for 1st dose of vaccination. Group (1) vaccinated with FMD serotype O; Group (2) vaccinated with FMD serotype A, and group (3) vaccinated with serotype SAT2.

Each vaccine was inoculated I/M in a dose of 1ml/hen. A second dose of vaccination was emulsified in Freund's incomplete adjuvant after 2 weeks receiving a booster dose after 4weeks of first vaccination. Blood samples from hens in each group were taken after 2 weeks of last vaccination. The eggs from the three groups were collected daily after 2 weeks of the last immunization and stored at 4^{o} C. The eggs yolk were separated, pooled and kept at -20^{o} C according to **Ikemori et al. (1997).** One hen group was kept without immunization as negative control.

IgY extraction and Purification

The method for IgY purification was adapted according to **Polson et al. (1980).** Briefly, the egg yolk was diluted 1:2 with sterile PBS (pH 7.4). For elimination of lipids and lipoprotein, 3.5% (wt/vol) polyethylene glycol (PEG) 6000 (Roth, Karlsruhe, Germany) was added. After gentle shaking followed by centrifugation (10,000 × *g* for 20 min at 4°C), the supernatant was decanted and solid PEG 6000 was added to a final concentration of 12% (wt/vol). This mixture was then centrifuged as above. The precipitate was dissolved in 10 mL of PBS, PEG was added to 12% (wt/ vol), and the solution was centrifuged. Finally, the precipitate was dissolved in 1.2 mL of PBS, transferred into a microdialysis device and dialyzed against PBS at 4°C.

The protein content (mg/mL) was measured photometrically at 280 nm and was calculated according to the Lambert-Beer law with an extinction coefficient of 1.33 for IgY. And also according to **Hoffmann and Richterich (1970)**.

Screening of chicken anti-FMDV sera and IgY:

Detection and evaluation of antibody titer against FMDV in hen sera and yolk samples were carried out by serum neutralization test (SNT) and Enzyme Linked Immune Sorbent Assay (ELISA) according to **Mettias et al. (1994)** and **Ikemori et al. (1997)** respectively.

Conjugation of FMD-IgY with horse radish peroxidase:

It was done according to **Hudson and Hay (1989**) who stated that, dissolve horse radish peroxidase 4mg/ml in distilled water, 200µl of sodium periodate solution freshly prepared and stir gently for 20 min. at room temperature, dialyse overnight at 4°C against sodium acetate buffer, add 20µl of sodium carbonate buffer to pH 9.5, and immediately add 1ml (8mg) of IgY protein to be conjugated. Leave at room temperature for 2 hours. After that add 100 µl of freshly prepared sodium borohydride solution (4mg/ml) in distilled water and leave for 2 hours at 4 °C, then dialysis against borate buffer.

Optimization of anti- IgY conjugate

The optimum titre of anti- IgY –HRP conjugate was determined by preparing several dilutions (1:5 - 1:1000) from it with PBS pH 7.4. Each dilution of conjugate was tested with the control positive and control negative antigen according to **OIE (2009**) and **Hiam et al., (2013)**.

Direct Sandwich ELISA test for FMD antigen serotypes detection.

It was done according to **McCullough et al., (1985**), ELISA plate were coated overnight at 4 °C with 100 µL of appropriated dilution (10µg/ml) protein concentration of rabbit antiserum homologous to antigen being used in carbonate buffer, the liquid phase of viral antigen samples prepared according to **Roeder and Le Blanc Smith (1987**) were added in 100 µl, and the modification applied in this method by addition of secondary antibody (chicken IgY conjugated with peroxidase) for each serotypes of FMD viral antigens.Washing the plated three times with (PBS+ tween 20). Then added 100 µl OPD substrate and incubation at room temperature in dark place for 10 to15 minutes till change of color from colorless to degree of yellow .after that stopping reaction by 50µl of stopping buffer. ELISA plate was reading by ELISA reader at 450 nm.

Virus neutralization test

It was done according to **OIE (2009**), and it performed for the quantitative FMD antibodies in sera of chicken and egg yolk IgY before conjugation.End point titer was expressed as the reciprocal Log_{10} dilution giving 50% of absorbance estimated according to method of spearman-Karber.

Real time polymerase chain reaction (RT-PCR):

It was done according **EL-Shehawy (2012)**

A-RNA extraction: RNA extraction was carried out using the QIAamp®Viral RNAkit (Qiagen, Germany) according to the manufacturer's protocol to all samples in a final volume of 50ml according to themanufacturer's instruction and stored at -80^{o}C until used.

B-Primers: Primer pair (PoR/PoF) for real time RT-PCR was synthesized by BioBasic, Canada. PoF (5´-CCTATG AGAACAAGC GCATC -3´) and PoR (5´-CAA CTT CTCCTG TAT GGT CC -3´) were derived from FMDV 3D polymerase for detection of FMDV and have no cross reaction with swine vesicular disease (Universal primer for FMDV).

C-Real-time RT-PCR (rRT-PCR): rRT-PCR was performed using QuantiTect® SYBR® Green RT-PCR Kit (Qiagen, Germany) as manufacturer's instructions. The cycling parameters were 50 °C for 30 min and 95 °C for 15min; then 30 cycles consisting of 94 °C for 15 s, 55°C for 30 s and 72 °C for 30 s. Negative control specimen was involved. Each sample was tested in duplicate. PCR amplification was carried out in the Thermo cycler Rotor-GeneQ (Qiagen, Germany)

Samples:

One hundred tongue epithelium samples; 50 OP fluid fluids and 20 nasal swabs were obtained from naturally infected cattle at Fayoum Governorate in addition to 100 tissue culture infected fluids were subjected to detect FMD viruses using Indirect solid phase Sandwich ELISA; Direct solid phase Sandwich ELISA using IgY conjugated with peroxidase specific for serotypes, and RT-PCR.

RESULTS

Table 1: FMDV type O, A and SAT2 antibody titers in chicken sera and egg yolk

WPI*	Mean FMDV serotypes antibody titers in chicken groups											
	Group (1)				Group (2)				Group (3)			
	Serotype (O)				Serotype (A)				Serotype (SAT2)			
	NT		ELISA		NT		ELISA		NT		ELISA	
	S	Y	S	Y	S	Y	S	Y	S	Y	S	Y
Pre- immunization	0	0	0.01	0.01	0	0	0.02	0.01	0	0	0.02	0.02
Day 0	0	0	0.02	0.01	0	0	0.02	0.02	0	0	0.02	0.01
1st Immunization with Freund's complete adjuvant												
1st week post 1st Immunization	0.91	0.90	1.29	1.27	0.92	0.91	1.3	1.28	0.92	0.91	1.29	1.27
2nd week post 1st Immunization (2nd Immunization with Freund's incomplete adjuvant)												
3rd week post 1st Immunization	1.35	1.34	1.66	1.35	1.34	1.67	1.65	1.35	1.36	1.34	1.68	1.67
4th week post 1st Immunization	1.52	1.51	1.8	1.8	1.55	1.53	1.82	1.81	1.54	1.53	1.81	1.80
4th week post 1st Immunization (Booster dose with Freund's incomplete adjuvant)												
5th week post 1st Immunization	2.2	2.15	2.6	2.55	2.25	2.23	2.6	2.58	2.25	2.24	2.62	2.60
6th week post 1st Immunization	2.50	2.58	2.82	2.8	2.61	2.6	2.83	2.81	2.6	2.59	2.81	2.80
Collection of eggs and storage at 4°C												

Group (1) vaccinated with FMD inactivated Monovalent O

Group (2) vaccinated with FMD inactivated Monovalent A

Group (3) vaccinated with FMD inactivated Monovalent SAT-2

*WPI: Week post immunization

Permissible result for FMDV SNT is 1.5 $\log_{10}$ & for ELISA OD is 1.8 - 1.85

Mean Permissible titer of IgY NT titer is 2.6 $\log_{10}$ and for ELISA OD is 2.8 - 2.83

Table 2: Sensitivity test of prepared FMD serotypes IgY conjugated with horse radish peroxidase against positive control of FMD serotypes antigens

FMD serotypes antigens	Dilution factor of IgY against serotypes	ELISA results
FMD serotype O	1:5	Positive
	1:25	Positive
	1:125	Positive
	1:250	Positive
	1:500	Positive
	1:1000	Negative
FMD serotype A	1:5	Positive
	1:25	Positive
	1:125	Positive
	1:250	Positive
	1:500	Positive
	1:1000	Negative
FMD serotype SAT-2	1:5	Positive
	1:25	Positive
	1:125	Positive
	1:250	Positive
	1:500	Positive
	1:1000	Negative
Control negative	1:5 for each anti-FMD serotypes IgY	Negative

Table 3: Evaluation of the prepared FMDV-IgY conjugated with horse radish peroxidase

Tested samples	Virus serotype	Percentage of positive samples using the applied tests		
		Traditional ELISA typing	RT-PCR	ELISA using the prepared IgY ELISA
100 Tongue epithelium	O	65	70	70
	A	10	15	13
	SAT2	5	7	7
	-ve	20	8	10
50 OP fluids	O	20	22	22
	A	13	13	13
	SAT2	13	14	14
	-ve	4	1	1
20 Nasal swabs	O	6	7	7
	A	4	5	5
	SAT2	3	3	3
	-ve	7	5	5

DISCUSSION

The definitive FMD diagnosis should be based on complex laboratory testing including indirect Sandwich ELISA and RT-PCR methods combined with virus isolation on cell culture (BHK, Pig or lamb kidney) as a gold standard method recommended by OIE. Depending on the great importance of FMD, accurate and rapid identification of the causative agent is an essential step in controlling the disease. This purpose needs the availability of specific diagnostic antiserum for each serotypes and kits which may be not available on request. So, the present work was designed to prepare anti-FMD (local types O, A and SAT2) IgY conjugated with horse radish peroxidase to saving time and cost where such IgY will be prepared in chicken egg yolk.

The yolk of eggs laid by immunized chickens has been recognized as an excellent source of polyclonal antibodies. Using chicken as the immunization host for producing egg yolk antibodies (IgY) instead of IgG from mammalian species brings a number of advantages: (1) the animal suffering is reduced (no bleeding), as antibodies are obtained directly from the egg and only egg collection is required upon immunization, (2) antibody isolation is fast and simple; (3) very low quantities of antigen are required to obtain high and long-lasting immunoglobulin titers in the egg yolk from immunized hens, and (4) a single egg contains as much antibodies as an average bleed from a rabbit **Davalos-Pantoja et al., (2000); Schade and Hlinak (1996); Tini et al., (2002**).

Using chicken as a host for antigen immunization and generation of polyclonal antibodies (IgY) has demonstrated several valuable advantages over existing mammalian systems as discussed by **Sock et al., (2012)**. The obtained results through the present work showed that chicken immunization with the monovalent FMD serotypes (O; A, and SAT-2) induced detectable antibodies against FMD serotype O, A and SAT2 by the first week post immunization as determined by VNT in chicken sera and egg yolk as 0.91 & 0.9; 0.92 & 0.91, and 0.92 & 0.91 for each serotypes respectively. And antibody titer against FMD serotypes measured by ELISA were 1.29& 1.27; 1.3& 1.28, and 1.29& 1.27 respectively .

These antibody titers against FMD serotypes were gradually increased by the 2nd immunization (4th week post the first immunization) as shown in table (1) recording their peaks for serotype O (2.5& 2.48 by VNT and 2.82 &2.8 by ELISA in chicken sera and egg yolk respectively; for serotype A (2.61& 2.6 by VNT and 2.83& 2.81 by ELISA, and for serotype SAT-2 (2.6& 2.59 by VNT and 2.81& 2.8 by ELISA) respectively. These findings agree with what reported by **OIE (2013)** concluded that such antibody titers are protective and **Gamal et al., (2015)** obtaining similar findings. Antibodies are useful for specifically recognizing antigens and the detection of antigen-antibody complex could be easy when precipitation occurs. Labeling of antibodies makes antigen identification easier.

As shown in (table 2) the sensitivity of prepared IgY specific for each FMD serotypes was 1/500 with specific control positive of FMD viral antigen serotypes Specific, rapid and sensitive serological tests like enzyme linked immune sorbent assay (ELISA) are required for accurate diagnosis of infectious agents to reach to well applicable control measures. It was concluded that ELISA was successful in the specific detection of FMDV from infected tissue culture and epithelial tissues. The ELISA compared favorably with the complement fixation test, being more sensitive and unaffected by anti-complementary factors **Crowther and Abu El-Zein, (1979)**. The use of an indirect technique of ELISA was efficient in detecting FMDV in cell culture fluids, mouse carcasses and cattle tongue epithelium.

The technique was also recommended for detecting serum antibody titers **Rai and Lahiri, (1981). Hamblin et al., (1986)** utilized ELISA for the detection of antibodies against FMD by titration of serum antibodies from more than 300 British uninfected and non vaccinated animals against the 7 immunologically distinct (FMDV) types. It was found that such Igy had a total protein value of 7.54, 7.2 and 7.4gm/dl for O, A and SAT2 respectively ; in this respect similar findings were obtained by **Manal and Mervat (2008)** who found that the immune globulin of the prepared FMD antiserum type-A was found to be 12.5gm⁄ dl. The obtained IgY FMD antibody titters (Table-1) were 2.58 and 2.80; 2.60 and 2.81; 2.81 and 2.80 by SNT and ELISA for type O; A and SAT2 respectively. These high FMD antibody titers came in agreement with those of **Barnett et al., (1996)** and **Abd El-Karim (2007)** who concluded that Mantonid oil ISA-206 has the ability to improve the immune response of vaccinated animals with FMD vaccine resulted in higher levels of immunity.

Such IgY was successfully conjugated with horse radish peroxidase and detection of the three types of FMD virus was carried out in 100 tongue epithelium samples; 50 OP fluid samples; 20 nasal swabs obtained from naturally infected cattle in addition to 100 tissue culture infected fluids using traditional ELISA; RT-PCR and ELISA using the prepared conjugated IgY (Table-2). The results of three techniques came in a parallel manner confirming each other. So the prepared FMD IgY conjugated with horse radish peroxidase could be considered of diagnostic benefit in detection and typing of FMD virus providing local preparation available on request. And from economic value, Direct Sandwich ELISA where used one type only of secondary antibody (IgY conjugation against each serotype) while Indirect Sandwich ELISA need for anti-guinea pig as secondary antibody and also anti-anti-guinea pigs labeled by peroxidase.

CONCLUSION

It can be concluded that, the prepared FMD IgY conjugated with horse radish peroxidase could be considered of diagnostic benefit in detection and typing of FMD virus providing local preparation available on request.

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