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DIFFERENTIATION BETWEEN FOOT AND MOUTH DISEASE VIRUS INFECTED AND VACCINATED CATTLE USING RECENT TECHNIQUES

(With One Table and One Figure)

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التمييز بين الأبقار المحصنة والمصابة بفيروس مرض الحمى القلاعية باستخدام التقنيات الحديثة

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لدراسة إمكانية التمييز بين الحيوان المحصن والحيوان المصاب بفيروس مرض الحمى القلاعية تم استخدام مائة بقرة، سبعون منها غير محصنة ضد فيروس مرض الحمى القلاعية وثلاثون محصنة. هذه الحيوانات تم فحصها من الناحية الإكلينيكية كما تم فحصها باستخدام نوعين من اختبار الإليزا، النوع الأول (اختبار الإليزا المزدوج الغير مباشر) تم استخدامه للتعرف على فيروس مرض الحمى القلاعية وتحديد عترته أما النوع الثانى (اختبار الإليزا القياسى) تم استخدامه للتعرف على الأجسام المناعية المضادة للبروتينات غير البنائية لفيروس مرض الحمى القلاعية (الموجودة فى الفيروس النشط المسبب للعدوى فقط وغير موجودة فى الفيروس المستخدم فى التحصين). الفحص الإكلينيكى أظهر معاناة ١٤ بقرة غير محصنة من إفرازات لعابية، التهابات فى الفم، عرج وارتفاع فى درجة الحرارة. بعد إجراء اختبار الإليزا المزدوج الغير مباشر تم تسجيل ثلاث عترات من فيروس مرض الحمى القلاعية، العترة O تم التعرف عليها فى ١٦ بقرة غير محصنة و ٢١ بقرة محصنة، العترة A تم التعرف عليها فى ٧ بقرات غير محصنة ، العترة C تم التعرف عليها فى بقرتين غير محصنتين. اختبار الإليزا القياسى أوضح وجود خمس أنواع من الأجسام المناعية المضادة للبروتينات غير البنائية لفيروس مرض الحمى القلاعية 3ABC-3D-3A-2C- Lb). تم التعرف على هذه الأجسام المناعية المضادة لجميع أنواع البروتينات غير البنائية للفيروس ما عدا النوع الأول (Lb) فى ٢٥ بقرة غيرمحصنة من الذين ثبت إصابتهم بفيروس مرض الحمى القلاعية مع اختبار الإليزا المزدوج الغير مباشر بينما تم التعرف على الأجسام المناعية المضادة للبروتين غير البنائى(Lb) فى ١٦ بقرة غير محصنة كما ثبت أن هذه الأجسام المناعية موجودة عند الإصابة بأى عترة من عترات الفيروس الثلاثة التى تم التعرف عليها فى هذه الدراسة (C:A:O) مما يؤكد عدم وجود علاقة بين عترة الفيروس ووجود البروتينات غير البنائية للفيروس وهذا يجعل استخدام هذه التقنية فى التمييز بين الحيوان المحصن والحيوان المصاب غير مقيد بعترة الفيروس. فى نفس الوقت كانت كل

الحيوانات المحصنة سلبية لهذا الاختبار. أثبتت هذه الدراسة أن اختبار الإليزا المزدوج الغير مباشر الذى تم استخدامه فى التعرف على فيروس مرض الحمى القلاعية غير قادر على التفريق بين الحيوان المحصن والحيوان المصاب بفيروس مرض الحمى القلاعية حيث أنه أعطى نتائج إيجابية مع الحيوانات المحصنة والحيوانات المصابة بالفيروس لهذا يوصى هذا العمل باستخدام اختبار الإليزا القياسى الذى يعتمد على التعرف على الأجسام المناعية المضادة لأى نوع من البروتينات غير البنائية لفيروس مرض الحمى القلاعية (ماعدا البروتينLb الذى تم التعرف عليه فى بعض الحالات المصابة وليس كلها) حيث أنه اختبار حساس لتشخيص الإصابة بفيروس مرض الحمى القلاعية حتى فى الحيوانات التى لاتعانى من أى أعراض بالإضافة إلى أنه ناجح تماما فى التمييز بين الحيوان المحصن والحيوان المصاب بفيروس مرض الحمى القلاعية وهذه خطوة هامة جدا للسيطرة على مرض الحمى القلاعية فى مصر. هذه أول دراسة تتناول التمييز بين الحيوان المحصن والحيوان المصاب بفيروس مرض الحمى القلاعية كما أنها أول دراسة تبحث فى التعرف على الأجسام المناعية المضادة للبروتينات غير البنائية لفيروس مرض الحمى القلاعية فى مصر بالإضافة إلى أنها أول عمل يدرس العلاقة بين البروتينات غير البنائية وعترة فيروس مرض الحمى القلاعية.

SUMMARY

To study the possibility of differentiation between foot and mouth disease virus (FMDV) vaccinated and infected cattle, 100 cattle were used, 70 of them were non vaccinated while the remainder 30 were vaccinated against FMDV. These animals were examined clinically and investigated with two forms of enzyme linked immunosorbent assay (ELISA). The 1st form (indirect sandwich ELISA) was used for detection and serotyping of FMDV while the 2nd form (standard ELISA) was used to identify antibodies against non-structural proteins of FMDV (present in active FMDV that responsible for infection and absent in vaccination virus). Clinical examination showed that 14 cattle were suffered from salivation, lameness and rise of body temperature while the remainder of cattle were apparently healthy. By using indirect sandwich ELISA, three serotypes (O,A and C) of FMD virus were reported. Serotype O was detected in 16 non vaccinated and 21 vaccinated cattle, serotype A was detected in 7 non vaccinated cattle and serotype A was detected in 2 non vaccinated cattle. After performing standard ELISA five types of antibodies against FMDV non structural proteins (Lb, 2C, 3A, 3D and 3ABC) were recorded. All of these antibodies (except antibodies against non-structural protein Lb) were observed in 25 non vaccinated cattle while antibodies against non structural protein Lb were observed in 16 non vaccinated cattle only, all of these cattle w proved to be positive for FMDV by indirect sandwich ELISA. The study proved that there is no relation between FMDV serotype and presence of FMDV non structural

protein as antibodies against non structural proteins were detected in animal infected with any of three FMDV serotypes recorded in the study. Results of the present work proved that indirect sandwich ELISA could use in detection and typing of FMDV but could not use in differentiation between FMDV vaccinated and infected cattle as it gave positive results with both animals. At the same time, standard ELISA used successfully in differentiation between FMDV vaccinated and infected cattle and gave positive results with infected cattle only. Therefore, our study recommend using standard ELISA based on detection of any type of antibodies against non structural proteins of FMDV (except non-structural protein Lb which recorded in some but not all infected animals) as it is sensitive technique in diagnosis of FMD even in apparently healthy animals and it is capable of differentiation between FMDV vaccinated and infected animals successfully. Differentiation between vaccinated and infected cattle is an important step in control of FMD in Egypt. This is the first study investigate differentiation between FMDV vaccinated and infected animal, the first to identify antibodies against non structural proteins of FMDV in Egypt and the first to study the relation between FMDV serotypes and presence of FMDV non structural proteins.

Key words: *Foot and mouth disease (FMD), Foot and mouth disease virus(FMDV), ELISA, Non-structural proteins, cattle, Egypt*

INTRODUCTION

Foot and mouth disease (FMD) continues to be one of the most feared animal diseases in the world. Although the mortality rate caused by FMD is generally low, the disease is taken seriously because of its resulting high morbidity. Production losses, trade restrictions, and costly eradication and vaccination programs make it a disease that most countries would like to eradicate (Fondevila *et al.*, 1995; Sorensen *et al.*, 1998a; Baipoledi *et al.*, 2004; Taylor *et al.*, 2004). In the Middle East FMD is endemic (Hafez *et al.*, 1993a;Callens *et al.*, 1998).

FMD is caused by RNA virus of the genus Aphthovirus, Picornaviridae family. Seven immunologically distinct serotypes of the virus have been identified (Grubman and Baxt, 2004; Musser, 2004)

At present, identification and typing of FMD virus largely relies on serological tests (Nunez *et al.*, 1998). Serological testing for antibodies against FMD virus is hampered by the existence of 7 different serotypes. At the same time, current serological tests do not distinguish

between vaccinated and infected animals (Sorensen *et al.*, 1998b; Shen *et al.*, 1999)

It has been shown that tests based on non-structural proteins are capable of identifying antibodies against all seven serotypes of FMD virus (Bronsvoort *et al.*, 2004a; Moonen *et al.*, 2004). The presence of antibodies against structural proteins of FMD virus proves that the animal had contact with FMD viral antigen either due to infection or vaccination. The detection of non-structural proteins gives evidence of infection and is used to distinguish animals that has been infected from those that have been vaccinated (Callens *et al.*, 1998; Dekker and Gijsen, 1998; Foster *et al.*, 1998; Mackay, 1998).

Differential diagnosis of animals which have been infected with FMD virus from those that have been vaccinated is important because the first group can become carrier of the virus and thus a potential source of outbreaks of the disease. Because current serological tests can not distinguish between the two groups, attention has been focused on alternative method of differentiation (Mezencio *et al.*,1998; Sorensen *et al.*, 1998a). However, assays detecting antibodies against non-structural proteins have the potential to differentiate infected animals from those that have been merrely vaccinated (Lubroth *et al.*, 1998; Brocchi *et al.*,1998; Malirat *et al.*,1998; Bronsvoort *et al.*, 2004b).

FMD virus may be excreted several days before classical symptoms appear. Considering the highly contagious nature of FMD and the ability of FMD virus to establish persistent infection, there is a need to establish a rapid test for diagnosis of FMD and estimation of subclinical infection of FMD. This test will help and should be an important step in disease control (Bergmann *et al.*,1998; Marquardt and Haas, 1998).

In Egypt, there is no work concerning differentiation between FMD virus infected and vaccinated cattle therefore, the aim of this study was to differentiate cattle infected with FMD virus from that vaccinated against FMD virus by detection of antibodies against non-structural proteins of the virus. Study the correlation between serotype of FMDV and non-structural proteins was an another important aim of the present work.

MATERIALS and METHODS

Animals:

100 cattle (2-4 years old) were used in the study, 70 of them were non vaccinated against FMDV and 30 were FMDV vaccinated.

Blood:

5 ml whole blood was collected from all investigated cattle and used for indirect sandwich ELISA.

Serum:

5 ml blood was collected from each examined cattle to obtain serum for standard ELISA.

Clinical examination:

All cattle used in this study examined clinically. An evaluation system modified from that of Carpenter *et al.*, (2004) was used. Specific clinical signs were reported.

Indirect sandwich ELISA for detection and typing of FMD virus:

1- Coating of microplates

50 µl of 1/1000 diluted rabbit trapping antibody was transferred to each plate well. Rows A to H of microplate received respectively antisera to serotypes O,A,C,SAT1,SAT2,SAT3, Asia1 and normal non immune serum. The plates were covered with lids and incubated at 39° C for 1 hour or left to coat over night at 1-8° C. The contents of all antibody coated miceroplates were removed and the plates were washed by wash buffer using automatic plate washer wellwash4 (Labsystems, A Thermo Bioanalysis Company, Research Technology Division, Helsinki, FINLAND).

2- Addition of tested samples and control antigen

Wells of columns 1-6 were loaded with diluent buffer A. To well 1 of row A of microplate 12.5 µl of control antigen type O was added. The same step was repeated in well 1 of rows B to H for control antigen types A,C,SAT1,SAT2,SAT3, Asia 1and negative control antigen respectively. To obtain a five-fold dilution, the content of wells were mixed by multichannel pipette and 12.5 µl was transferred from well 1 to 2 (rows A to H), 12.5 µl was transferred from well 2 to 3, 12.5 µl was transferred from well 3 to 4 and 12.5 µl was discard from well 4 (rows A to H). The remainder of the plate was loaded test sample, 50 µl of 10% original sample. Sample 1 was added to wells 7,8 of rows A to H, the second sample was placed in wells 9,10 of rows A to H and the third sample was placed similarly in wells 11,12 of rows A to H. The microplates were covered with lids and placed on the shaker at 39°C for 1 hour.

3- Addition of Detecting Antibodies

Before the end of the test sample and control antigen incubation, prepare working dilution of the blocked guinea pig detection sera in diluent buffer B (0.01 M phosphate buffered saline. pH 7.4 plus 0.05%

Tween 20 plus 5% w/v skimmed milk powder). Wash the plate with wash buffer three times. Transfer 50ul volumes of each guinea pig serum 1/100 dilution to each plate well in the appropriate order to rows A to H which receive antisera to serotypes O,A,C,SAT1,SAT2,SAT3,Asia1 and normal non immune serum respectively. Cover plates with lids and incubate at 39° C for 1 hour.

4- Addition of Conjugate

Immediately before the end of the detecting antibody incubation, prepare a working dilution (1/200) of the conjugate in diluent buffer B. Wash the plate three times. Add 50 µl to all wells of rows A to H. Cover plates with lids and incubate at 39°C for 45 minutes.

5- Addition of Substrate/Chromogen and stopping solution

Immediately before the end of conjugate incubation, prepare the substrate / chromogen solution. For one plate dilute 30 µl of substrate stock (H2O2) in 6 ml of chromogen stock solution (OPD). Wash the plate three times. Immediately after washing add 50 µl of the substrate/chromogen solution to the wells of the microplates. Immediately begin timing after filling of the first well and incubate at room temperature for 15 minutes. Add 50 µl of the stopping solution (1.25 M sulphuric acid)

6- Calculation and interpretation of results

Place the microplate in the carriage of the multiskan (Labsystems, A Thermo Bioanalysis Company, Research Technology Division, Helsinki, FINLAND) to measure the optical density (OD) of the samples and control at 450 nm.

$$\text{The mean background of each row} = \frac{\text{Optical density (OD) value of well 5+OD of well 6}}{2}$$

Corrected OD value = Actual OD for each serotype (reading of multiskan) – mean Background of the row

- A mean corrected OD value of > 0.1 above background indicate positive result. Values close to 0.1 should be confirmed by retesting that sample.
- All reagents used in this test were provided by Institute for animal health, Pirbright, United Kingdom (FAO/OIE world and reference laboratories)
- This test was performed according to manufacturer's direction and as described by Hafez *et al.*, (1993b) and Bronsvoort *et al.*, (2004a).

Standard ELISA for detection of antibodies against non structural proteins of the virus:

1- Preparation of the reagents

a- All reagents were allowed to equilibrate the room temperature (20° C-25° C).

b- 10x concentrated washing solution was diluted 1:10 with distilled water.

2- Preparation of samples and controls

Each sample, positive and negative control was diluted 1:100 with supplied diluent.

3- Addition of samples and controls

100 µl of pre-diluted sample, positive and negative control were added to the appropriate wells of the microtiter plate as follow

(a) Columns 1 and 2 were coated with non-structural protein Lb
(b) Columns 3 and 4 were coated with non-structural protein 2C
(c) Columns 5 and 6 were coated with non-structural protein 3A
(d) Columns 7 and 8 were coated with non-structural protein 3D
(e) Columns 9 and 10 were coated with non-structural protein 3ABC
(f) Columns 11 and 12 were coated with non-structural protein Lb
(g) Row A was specified for positive controls
(h) Row G was specified for negative controls
(i) Other rows were specified for samples

4- Incubation of the microtiter plate

The microtiter plate was covered with a lid and incubated for 60 minutes at 37°C in a humid chamber

5-Washing of the micotiter plate

After incubation, the microtiter plate was washed three times with 1:10 diluted washing solution using automatic washer wellwash4 (Labsystems, A Thermo Bioanalysis Company, Research Technology Division, Helsinki, FINLAND)

6- Addition of conjugate

100 µl Anti-ruminant-IgG-PO-Conjugate was dispensed into each well, the plate was covered and incubated for 60 minutes at 37°C in a humid chamber

7- Repeat step 5

8- Addition of Substrate

100 µl TMB-Substrat was dispensed into each well, the plate was incubated at 25 °C for 15 minutes.

9- Reading of the results

The reaction was stopped by addition of 100 µl stopping solution to each well. The results were read using a multiskan (Labsystems, A Thermo Bioanalysis Company, Research Technology Division, Helsinki, FINLAND) at a wavelength of 450nm.

10- Interpretation of results

In order to validate the assay, the optical density of the (OD) of the positive control should not exceed 2.0, the OD of negative control should not exceed 0.5 and the difference between the positive and negative control must be _> 0.4

$$\text{Value \%} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}}$$

Table 1: Interpretation of results of standard ELISA.

Value	< 20%	20 – 30%	> 30%
Interpretation	Negative	Ambiguous	Positive

This technique was performed according to instructions of manufacturer (Bromeli Diagnostics, Intervet, Germany) and as described by De Diego *et al.* (1997) and Clavijo *et al.* (2004).

RESULTS

Clinical examination:

14 cattle were suffered from salivation, lameness and rise of body temperature while the remainder of examined cattle (86) were apparently healthy.

Indirect sandwich ELISA:

- Three FMD virus serotypes were reported (O,A andC)
- Serotype O observed in 16 non vaccinated and 21 vaccinated cattle
- Serotype A observed in 7 non vaccinated cattle
- Serotype C observed in 2 non vaccinated cattle

Standard ELISA:

- Five types of antibodies against five types of FMD Viral non-structural proteins (Lb,2C,3A,3D,3ABC) were reported.
- Antibodies against FMD Viral non structural proteins 2C,3A,3D,3ABC were detected in 25 non vaccinated cattle cattle.
- Antibodies against FMD Viral non structural protein Lb was detected in 16 non vaccinated cattle.

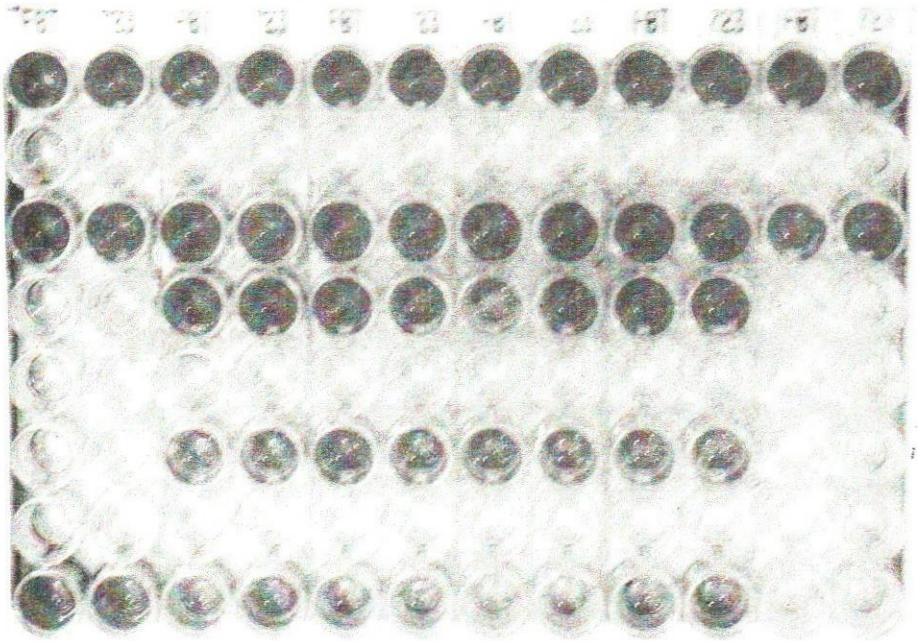

Fig. 1: Results of standard ELISA for detection of antibodies against non-structural proteins of FMDV.

* Two columns were used for each type of examined antibodies.

(A) Row A 1-12 positive control for antibodies against non-structural proteins Lb, 2C, 3A,3ABC and Lb respectively.

(B) Row B 1-1-0 (negative results for Lb, 2C, 3A, 3D and 3ABC respectively in one sample),row C11-12 (negative result for Lb in another sample).

(C) Row C1-10 (positive results for Lb, 2C, 3A, 3D and 3ABC respectively in one sample) ,row C11-12 (positive result for Lb in another sample).

(D) Row D 1-2 (negative results for Lb), row D 3-10 (positive results for 2C, 3A, 3D and 3ABC respectively in one sample) and row D 11-12 (negative result for Lb in another sample).

(E) Row E 1-10 (negative result for Lb, 2C, 3A, 3D and 3ABC respectively in one sample) and Row E 11-12 (negative result for Lb in another sample).

(F) Row F 1-2 (negative result for Lb), Row F 3-10 (positive results for 2C, 3A, 3D and 3ABC respectively in one sample) and Row F 11-12 (negative result for Lb in another sample).

(G) Row G 1-12 negative control for antibodies against non-structural proteins Lb, 2C, 3A,3ABC and Lb respectively .

(H) Row H 1-10 (positive results for Lb, 2C, 3A, 3D and 3ABC respectively in one sample) and Row H 11-12 (negative result for Lb in another sample).

DISCUSSION

FMD is a highly contagious viral disease of cloven-hoofed animals. For several years, vaccination of animals, which had proven to be successful for the eradication of the disease, has been forbidden in the United States and the European Community because of the difficulty of differentiating between vaccinated and infected animals (Hohlich *et al.*, 2003).Multiplicity of FMD virus in animals poses a central problem in the policy of vaccination (Patil *et al.*, 2002).

Vaccination against FMD is a key element in the control of FMD. However, countries that vaccinate will have to reestablish their FMD free status. Because currently available vaccines stimulate the production of antibodies indistinguishable from those produced by infected animal in response to live virus and because vaccinated animals can be infected and become carriers of FMD virus, efforts have been made to develop diagnostic test that can differentiate vaccinated animals from those carriers and infected animals (Clavijo *et al.*, 2004).

The development of a serological test for FMD virus which is quick, easy to use and can differentiate vaccinated from convalescing or potential virus carriers would be a major advance in the epidemiological studies of FMD (Bronsvoort *et al.*, 2004a)

In the present study, to differentiate between FMD virus-infected and vaccinated animals, we used a serological assay capable of detecting antibodies against FMD virus non-structural proteins. An important objective in the epidemiology and control of FMD virus is the identification of infected animals and there is an international demand for a new diagnostic procedure with the precise objective of discriminating infected from vaccinated animal. The main immunological difference between infected and vaccinated cattle is that cattle exposed to replicating virus are exposed to viral non structural proteins. These non-structural proteins are not component of purified virus, therefore, vaccination or exposure of animal to non replicating virus should not stimulate immune responses to virual non-structural proteins. Thus detection of antibodies to non-structural proteins is very important to discriminate infected from vaccinated animals (Mackay, 1998; Moonen *et al.*, 2004).

In the present investigation we differentiated between infected and vaccinated animal by detection of five types of antibodies against non-structural proteins of FMD virus. Preliminary work on antibody responses to FMD virus non-structural proteins has been reported

suggesting that screening for antibodies against several rather than one non-structural protein may be a better indication to infection of the animal (Silberstein *et al.*, 1997; Foster *et al.*, 1998; Hohlich *et al.*, 2003; Sun and Wang, 2004)

In this study we used ELISA that proved to be sensitive. ELISA being easier to use, more robust and specific, and therefore offers an improvement for FMD virus antibody detection (Mackay *et al.*, 2001; Paiba *et al.*, 2004). Two forms of ELISA have been used in our work, one (indirect sandwich ELISA) for diagnosis and typing of FMDV and the other form (standard ELISA) for detection of antibodies to non-structural proteins of the virus .Bronsvoort *et al.*, (2004a) concluded that ELISA is quick, easy to use, can identify all seven serotypes of FMD virus, can differentiat vaccinated from infected animal and would be a major advance in epidemiological studies of FMD. However, indirect sandwich ELISA failed to differentiate between FMD virus vaccinated and infected animals, this result could be explained by the study of Clavigo *et al.*, (2004) who concluded that technique based on detection of FMD viral non-structural proteins is the only possible method for differentiation between FMD virus vaccinated and infected animals.

Immunological tests based on detection of antibodies against non-structural proteins could be used for the diagnosis of FMD virus infections and capable of differentiate between FMD virus -vaccinated and infected animals (Stilbrestein *et al.*, 1997). In our work we used standard ELISA for diagnosis of FMD viral infection as well as differentiation between FMD virus- vaccinated and infected cattle.

FMD virus and antibodies against non-structural proteins were detected in 11 apparently healthy cattle examined in our study. Musser (2004) concluded that FMD virus infected animals can become inapparent carrier. At the same time FMD virus serotype O was detected in 21vaccinated cattle only out of 30 examined FMD virus vaccinated cattle, this result could be explained by the fact that currently available FMD vaccine has an important disadvantages (Ishimaru *et al.*, 2004).

Three serotypes (A, O and C)of FMD virus were detected in our work. Abd El-Hakim and Abd El-Rahim (1999) recorded three serotypes (A.O an C) in Egypt by using polymerase chain reaction (PCR) assay. Antibodies against non-structural proteins examined in the present study were detected in animals infected with any of the three FMD virus serotypes (O,A and C) that reported in this study. Mackay *et al.*, (1998b) reported that all sera from cattle infected with any of the seven serotypes of FMDV were positive for antibody to non-structural proteins 2C, 3A,

3D and 3 ABC and the majority was positive to Lb. Therefore, the present work proved that there is no correlation between FMD virus serotype and presence or absence of non-structural proteins, so, test used in our investigation can be used in infection with any serotype of FMD virus without any restriction.

In the present investigation, antibodies against non-structural protein Lb was present in some but not all infected animal. At the same time, other non-structural proteins examined in this work (2C, 3A, 3D and 3 ABC) were detected in all infected animals. The same observation reported by Mackay *et al.*, (1998a). So we recommend using of ELISA used in this study for detection of any two of previously mentioned non structural proteins in diagnosis and differentiation between FMD virus infected and vaccinated animal with exclusion of non-structural protein Lb. This is the first study concerning differentiation between FMD virus infected and vaccinated animals,the first to identify antibodies against non-structural proteins of FMD virus in Egypt and the first to study the relation between FMD viral serotypes and non-structural proteins of FMD virus.

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