Application Of Recent Methods For Diagnosis Of Foot And Mouth Disease In Cattle And Buffaloes

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ABSTRACT

Egypt was recently exposed to a sever outbreak of Foot and Mouth Disease (FMD) in 2012 with a new introduced strain of the virus (SAT2). This study concerned with diagnosis of FMD by using some recent methods in addition to the traditional methods for diagnosis. A total of 326 animals were clinically examined in different localities at Sharkia Governorates; 301 animals showed clinical signs of FMD (this is the group that used in this study) and 25 animals were apparently healthy. The diseased animals consisted of 277 cattle and 24 buffaloes. Some of them were vaccinated with the FMD bivalent vaccine before the outbreak and others were not vaccinated. 82 clinical samples (73 epithelial tissues (Epi.T.) and 9 vesicular fluids (V.F)) from cattle and buffaloes were isolated on Baby Hamster kidney (BHK-21) cell line and 76 samples (92.7%) were positive. By using FMDV ELISA Kits for typing of the virus only serotype SAT2 was detected. Reverse transcriptase polymerase chain reaction (RT-PCR) was used for detection of SAT2 serotype in 10 sample collected from 5 diseased cattle and 5 diseased buffaloes from different localities. Only one sample showed negative reaction (this sample was also negative by virus isolation and ELISA typing). Serum samples were collected from the 301 animals at different times after the infection; the antibodies against FMDV non-structural protein were detected by FMDV'CHEK Test in 270 cases (89.7%) of examined animals while antibodies against the structural protein were detected by SNT and ELISA where antibodies against serotypes O, A and SAT2 were detected in 75.7%, 75.7% and 100% of examined animals for each serotype respectively by both tests. Mixed infection with more than one serotype was only recorded at Al-Salhia dairy farm.

It is concluded that combination between recent and traditional techniques for diagnosis of FMD help in accurate and rabid diagnosis and subsequently control of the disease.

Key words: FMD, Diagnosis, ELISA kits, RT-PCR, FMDV'CHECH Test

INTRODUCTION

Cattle constitute one of the major livestock species and an important economical factor in many countries. This species is heavily exposed to numerous viral diseases which drastically affect their wellbeing as well as their productivity. *(1).*

Foot-and-mouth disease (FMD) is a highly devastating and debilitating viral disease of the cloven-hoofed animals and considered as a serious threat to the livestock industry worldwide. The disease is caused by an RNA virus belonging to *genus Aphthovirus* of the *family Picornaviridae.* FMD virus exists in seven distinct serotypes including O, A, C, SAT 1, SAT2, SAT3, and Asia1 *(2).*

The disease is characterized clinically by high rise of temperature, appearance of vesicles

on the mouth, tongue, teats and the interdigital space of feet which results into off-feeding and lameness. Morbidity can reach 100% in a susceptible population while fatality rate of the disease is low except in young animals where mortality can reach 50% due to myocarditis *(3)*.

During 2012, there has been a dramatic u psurge in FMD outbreaks in Egypt that caused by FMDV serotype SAT2. The Egyptian authorities has reported a further 18 outbreaks of the disease. An estimated 6.3 million buffalo and cattle considered to be at risk in Egypt. *(4)*.

Conventional techniques for diagnosis of Foot and Mouth disease such as isolation of the virus, serum neutralization test (SNT) and enzyme linked immune sorbent assay (ELISA) are still in use for the routine detection of causative agents of different infectious diseases.

The development of research techniques involving Molecular Biology enabled the use of Reverse transcriptase polymerase chain reaction (RT-PCR) assay for the detection of FMD virus genetic materials in various biological samples. *(5,6)*

The viral RNA is translated in to a single polypeptide which is then cleaved into four structural protein (VP1,VP2,VP3 and VP4) that form the viral capsid, and eight nonstructural protein (L, 2A, 2B, 2C, 3A, 3B, 3C and a3D) that participate in viral replication and other interaction with the host cell (*7*). So detection of nonstructural antibodies indicates the previous virus infection, irrespective of vaccination status *(8,9)*.

The aim of this study planned to detect the clinical signs observed in cattle and buffaloes infected with FMD in addition to isolation, identification and typing of FMDV from clinical samples.

MATERIAL AND METHODS

Animals: 301 animals (277cattle and 24 buffaloes) with clinical signs lead to suspect FMD infection were examined in different localities at Sharkia Governorate (228 cattle from Al-Salhia dairy farm, 34 cattle from a farm at Menia Al- Kamh center, 17 buffaloes at Al-Qenayate center, 3 cattle at Abo Hamad center and 19 animals at the *Vet. Clinic all of them were not vaccinated except cattle at Al-Salhia dairy farm were regularly vaccinated with FMD local produced bivalent vaccine). Different clinical samples were collected from them for laboratory diagnosis (Table 1).

Clinical Samples: 73 epithelial tissues from buccal cavity (54 samples from cattle and 19 samples from buffaloes) and 9 vesicular fluids (7 samples from cattle and two samples from buffaloes) were collected from animals suspected of being infected with FMD at different times from the appearance of clinical signs of the disease on the animals. The samples were used for isolation of FMDV on BHK-21 cell line, typing of the virus by FMDV ELISA kits and detection of the virus by RT-PCR.

Serum Samples: Serum samples were collected from all diseased animals at different times from the appearance of clinical signs of the disease on the animals as in table (1). The samples were collected once from each animal. The samples were used for detection of antibodies against FMDV structural protein by serum neutralization test (SNT) and ELISA and against non-structural protein by FMDV'CHECK Test.

Table 1. Clinical samples collected from diseased animals

Localities	Species	No	diseased	Samples			Time of sample collection
				Epi. T.	V.F	Serum	
Al- Salhia farm	cattle	239	228	31	6	228	21 days after the onset of the disease
Menia El-kamh	cattle	34	34	10	---	34	12 days after the onset of the disease
Al- Qenayat	buffalo	20	17	12	2	17	8 days after the onset of the disease
Abo-Hamad	cattle	4	3	1	1	3	2 days after the onset of the disease
*Vet. Clinic	cattle	18	12	12	---	12	Different times
	buffalo	10	7	7	---	7	
Total	cattle	295	277	54	7	277	Different times
	buffalo	31	24	19	2	24	

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Tissue culture: Baby Hamster kidney cell line (BHK-21) Clone 13 maintained from FMD Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo using Eagl's medium with 8-10% sterile bovine serum as described by (*10*). It was used for virus isolation and SNT.

Preparation of virus: Epithelial tissue specimens were homogenized in a proper volume of tissue culture media with antibiotic, then clarified by centrifugation and filtrated via 0.22 µm Millipore filter and kept at -70°C until used for inoculation.

Isolation of FMD virus: Epithelial tissue suspension and vesicular fluid of suspected FMDV were inoculated onto (BHK-21) cell-culture. The tissue cultures were observed after 24 hrs and 48 hrs for the cytopathic effect (CPE) of FMDV (*11*).

FMDV ELISA Kits for antigen detection provided by the FMD World Reference Laboratory (WRL-Pirbright, UK) (*12*).

Detection of FMD virus genome

RNA was extracted from clinical samples. All the extracted RNAs were tested by one-step RT-PCR using aprimer set as

FMDV-3RAGCTTGTACCAGGGTTTGGC.

CommonSAT257-3FGGCGTTGAGAAACAACTGTG.

Serological study

A-Evaluation of FMDV infective status: Serum samples were used to monitor the antibody against FMDV non-structural protein by FMDV'CHECK Test for detection of the infected animals irrespective to vaccination status.

B-Serum neutralization test: Collected sera were examined by serum neutralization test for detection of FMD antibodies according to (*13*).

C-Direct ELISA: used for detection of FMDV specific antibodies according to (*14*).

FMDV: The FMDV strains used in this study were FMDV strain O PANASIA 2, A/IRAN-05 and SAT2/EGY/2012. They were supplied by FMD Department (Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo).

RESULTS

Clinical examination of the animals

A total of 326 animals (295 cattle and 31 buffaloes) were clinically examined and there were 301 animals (92.3%) showed typical clinical signs of FMD (277 cattle (93.9%) and 24 buffaloes (77.4%)) and 25 animals were apparently healthy. At Al-Salhia dairy farm and Menia Al-Kamh dairy farm there were deaths in all the newly born animals during the outbreak of the diseases in 2012. Only the group of diseased animals was used in the present study.

Isolation of FMDV

FMDV was isolated from 67 epithelial tissue samples (91.8%) out of 73 tissue samples and from all vesicular fluid samples with a percent of 100% (Table 2). The positive samples showed clear CPE on BHK-21 at 48h after inoculation in the form of aggregation, lysis and detaching of some cells.

Detection and typing of FMDV by FMDV ELISA Kits

From total examined 73 Epi.T.; 67 samples were positive to antigen ELISA serotyping while all V.F. were positive. Only serotype SAT2 was detected.

Table 2. Isolation of FMDV on BHK-21 cell line

Localities	Year	Species	Samples					
			Epi. T.			V.F		
			No	+ve	%	No	+ve	%
Al- Salhia dairy farm	2012	Cattle	31	29	93.5	6	6	100
Menia El-kamh	2012	Cattle	10	10	100	---	---	---
Al- Qenayat	2013	Buffalo	12	12	100	2	2	100
Abo-Hamad	2012	Cattle	1	1	100	1	1	100
Vet. Clinic	2011	Cattle	3	2	66.6	---	---	---
	2012	Cattle	4	4	100	---	---	---
		Buffalo	5	3	60.0	---	---	---
	2013	Cattle	5	4	80.0	---	---	---
		Buffalo	2	2	100	---	---	---
Total		Cattle	54	50	92.6	7	7	100
		Buffalo	19	17	89.5	2	2	100

Table 3. Detection and typing of FMDV by FMDV ELISA Kits.

Clinical samples		No	+Ve	Antigen ELISA serotyping of isolated virus						
				O	A	C	SAT1	SAT2	SAT3	ASIA 1
Epi. T		73	67	-ve	-ve	-ve	-ve	+ve	-ve	-ve
V.F.		9	9	-ve	-ve	-ve	-ve	+ve	-ve	-ve
+Ve Control	O	1	1	+ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	1	1	-ve	+ve	-ve	-ve	-ve	-ve	-ve
	C	1	1	-ve	-ve	+ve	-ve	-ve	-ve	-ve
	SAT1	1	1	-ve	-ve	-ve	+ve	-ve	-ve	-ve
	SAT2	1	1	-ve	-ve	-ve	-ve	+ve	-ve	-ve
	SAT3	1	1	-ve	-ve	-ve	-ve	-ve	+ve	-ve
	ASIA1	1	1	-ve	-ve	-ve	+ve	-ve	-ve	+ve
-ve control		1	0	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Detection of FMDV serotype SAT2 by RT-PCR.

FMDV serotype SAT2 was detected in 7 epithelial tissue sample out of 8 samples and from the two used V.F samples. The negative sample was also negative for virus isolation and ELISA serotyping.

Serological study

Detection of antibodies against FMDVNon-structural protein NSP

Nonstructural antibodies were detected in 270 animals out of 301 animals (89.7%) and 31 cases were negative (10.3%), (Table 4).

Table 4. Detection of FMDV serotype SAT2 by RT-PCR

No	species	Localities	Type	Detection of serotype SAT2	
				+ve	%
1	Cattle	Al-Salhia	Epi. T.	+ ve	
2	Cattle	Menia Al-Kamh	Epi. T.	+ ve	
3	Cattle	Abo-Hamad	Epi. T.	+ ve	
4	Cattle	Vet. Clinic	Epi. T.	+ ve	87.5
5	Buffalo	Vet. Clinic	Epi. T.	- ve	
6	Buffalo	Vet. Clinic	Epi. T.	+ ve	
7	Buffalo	Al- Qenayat	Epi. T.	+ ve	
8	Buffalo	Al- Qenayat	Epi. T.	+ ve	
9	Cattle	Al –Salhia	V.F.	+ ve	100
10	Buffalo	Al- Qenayat	V.F.	+ ve	

Photo 1. Detection of FMDV serotype SAT2 by RT-PCR.

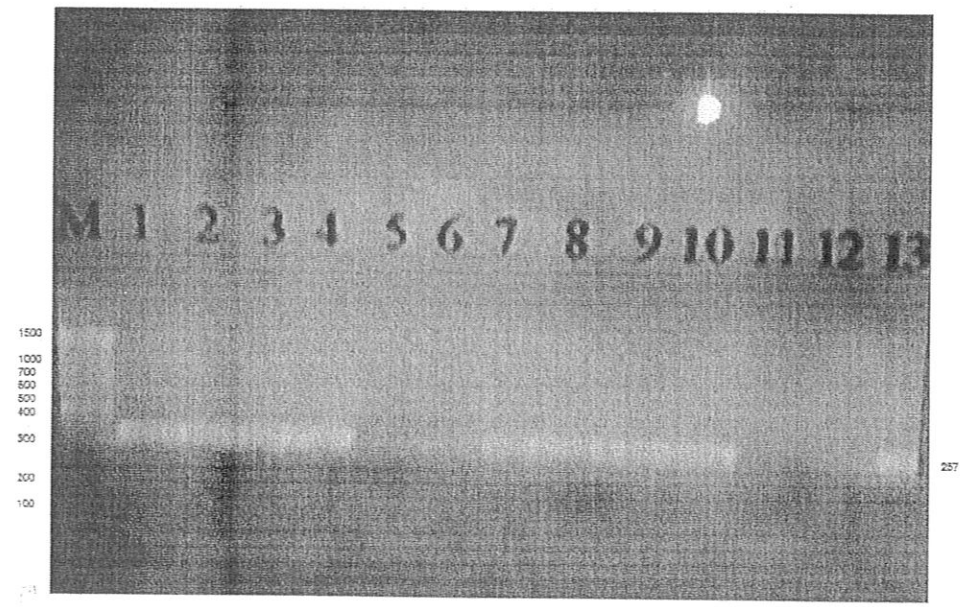

Photo. 1. Detection of FMDV genome by RT-PCR. Common SAT 257-3F Primer was used for targeting 1D coding region of the virus. M: 1500 bp DNA ladder. Lanes 1 2, 3, 4, 6,7,8,9&10 : positive FMDV isolates (257bp); lane 5: negative specimen; Lane 11 negative control and 12&13 positive control

Table 5. Results of detection of antibodies against FMDV nonstructural protein in different localities

Localities	No	Positive		Negative	
		No	%	No	%
Al- Salhia farm	228	203	89.04	25	10.96
Menia El-kamh	34	34	100	0	0
Al- Qenayat	17	17	100	0	0
Abo-Hamad	3	0	0	3	100
Vet. Clinic	19	16	84.2	3	15.8
Total	301	270	89.7	31	10.3

Detection of antibodies against FMDV structural protein by SNT and ELISA.

Serum samples from examined animals were tested by SNT and ELISA.Antibodies against FMDV serotype O and A were detected only at Al-Salhia dairy farm by both tests. The mean antibodies titre for serotype O was 2.1 $\log_{10}TCID_{50}$, for serotype A it was 2.4 $\log_{10}TCID_{50}$ `and for serotype SAT2 it was 1.68 $\log_{10}$ $TCID_{50}$ by SNT. In ELISA the mean antibodiestitre for serotype O,A and SAT2 was 2.52, 2.89and 1.98 $\log_{10}$ $TCID_{50}$for each serotype respectively.

Table 6. Detection of antibodies against FMDV by SNT and ELISA

Localities	SNT						ELISA					
	O		A		SAT2		O		A		SAT2	
	+VE	%	+VE	%	+VE	%	+VE	%	+VE	%	+VE	%
Al- Salhia farm(228)	228	100	228	100	228	100	228	100	228	100	228	100
Menia El-kamh (34)	0	0	0	0	34	100	0	0	0	0	34	100
Al- Qenayat (17)	0	0	0	0	17	100	0	0	0	0	17	100
Abo-Hamad (3)	0	0	0	0	3	100	0	0	0	0	3	100
Vet. Clinic (19)	0	0	0	0	19	100	0	0	0	0	19	100
Total (301)	228	75.7	228	75.7	301	100	228	75.7	228	75.7	301	100

DISCUSSION

The diseased animals showed severe rise in body temperature, ropy salivation and vesicles in mouth that then ruptured forming ulceration in the entire buccal cavity. The condition was related to feverial state as well as the tropism of FMDV to the epithelium of the gastrointestinal tract (*15*).

High mortality rate in young animals reached to 100% in the newlyborne. The mortality in calves usually attributed to acute or hyper-acute myocarditis (*16*).

Clinical signs of FMD in this study were sever and clinically infected animals showed typical clinical signs of infection due to the outbreak that occurred in February 2012

with serotype SAT2. There was no antigenic immunity in the animals against this strain as FMDV serotype A and O were the main causes of the disease in Egypt and SAT2 had not been recorded since 1950 and the used vaccine was bivalent vaccine against A and O strains *(4)*.

Inoculation of 73 epithelial tissues suspension and 9 vesicular fluid samples on BHK-21 cell lines revealed that FMDV was isolated from 67 tissue samples (91.8%) and all V.F samples (100%). Nearly similar results were detected by *(17,18)* who isolated FMDV from all clinical samples collected from different Egyptian governorates during the outbreak of FMD in 2012.

A lower percentage was detected by *(19)* in Egypt who detected a percentage of 73.2% of positive cases. Also *(20)* in Pakistan recorded 55.2% as a percent of positive samples that showed CPE of FMDV in tissue culture at 48h after inoculation.

This variation may be due to the difference of the disease pattern during which the samples were collected and the difference in the localities from which samples were collected.

Isolated FMDV was typed by FMDV ELISA Kits andonly serotype SAT2 was detected. The same results were recorded by *(21)*they recorded that only FMDV serotype SAT2 was detected during the outbreak of the disease in 2012. This indicates that FMDV serotype SAT2 was the main cause of the disease in 2012.

Using RT-PCR for detection of FMDV serotype SAT2 revealed bands of 257 bp in comparison with the ladder marker were detected in 7 samples out of 8Epi. Tissues while both V.F. samples were positive. *(22)* recorded that only FMDV serotype SAT was isolated from samples collected during the outbreak of the disease in 2012. Also *(23)* detected serotype SAT2 during the same outbreak.

By testing the serum samples collected from diseased animals (301 samples) against FMDV NSP antibodies by FMDV'CHECK Test the obtained results revealed 89.7% of the examined samples with positive reaction on the strip. Nearly similar seroprevalence NSP antibodies was detected by *(24)* where they reported 85% as a seroprevalence to FMD NSP antibodies While *(25,26)* in Egypt reported lower seroprevalence of antibodies against NSP with 30.3% and 38.9% for each study respectively.

The high percent of NSP antibodies in present study may be interpreted as this study mostly was performed during an outbreak of a new strain of FMDV so most animals acquired the infection with this strain and the samples were collected from diseased animals while the two studies performed in Egypt were somehow away from the time of the outbreak and they collected a random samples.

There were 25 cattle in Al-Salhia farm, three cattle at Abo- Hamad center and three animals at the veterinary clinic showed clinical signs of FMD but the antibodies against FMDV NSP were not detected by the test. *(27)*found similar cases during their study.

The findings suggest that the result obtained were not due to poor sensitivity in the FMDV'CHECK Test but reflected the fact that antibodies to NSP are first detected at 8-10 days post infection and reach the maximum levels at about 20 days post infection and demonstrable level of the antibodies can be detected at least 1 year post infection *(8)*, *(27)*.Serum samples from these animals were collected at 2-3 days post infection.

During detection of antibodies against structural protein of FMDV by using SNT and ELISA, the results revealed that antibodies against serotype SAT2 was detected in all diseased animals with a percentage of 100%. At Al-Salhia farm antibodies against serotype A and O were detected in all animals as the farm was periodically vaccinated with the local FMDV bivalent vaccine. While at the other localities only antibodies against serotype SAT2 were detected with a percentage of 100% as the animals were not previously vaccinated.

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الملخص العربى

تطبيقات للوسائل الحديثة فى تشخيص مرض الحمى القلاعية فى الابقار والجاموس

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يعتبر مرض الحمى القلاعية من اخطر الإمراض التى تصيب المجترات وقد تعرضت مصر مؤخرا لوباء شديد من هذا المرض أسفر عن خسائر عدة فى الثروة الحيوانيه٠فى هذا الدراسة تم استخدام بعض التطبيقات الحديثة بالاضافه الى الطرق التقليدية قى تشخيص هذا المرض. تم فحص ٣٢٦حيوان من مناطق مختلفه فى محافظة الشرقية فحصا إكلينيكيا وق أوضحت النتائج ظهور الإعراض المثالية للمرض على ٣٠١ حيوان وهى المجموعة التى تم استخدامها فى هذه الدراسة. تم عزل فيروس مرض الحمى القلاعية من عينات مرضية على المزارع النسيجية (كلى الفئران الرضيعه) وتم تحديد نوع العتره المسببة للمرض باستخدام اختبار Antigen ELISA وكانت هى عتره SAT(2) كما تم تشخيص هذه العتره باستخدام تفاعل البلمرة المتسلسل العكسى٠

تم تحديد الأجسام المضادة لعترات (O, A , SAT2) فى مصل دم الحيوانات التى تم فحصها باستخدام اختبار الاليزا واختبار مصل الدم المتعادل واسفرت النتائج عن وجود أجسام مضادة ضد العترات الثلاث بكلى الاختبارين٠