Global Veterinaria 12 (5): 660-666, 2014
ISSN 1992-6197
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DOI: 10.5829/idosi.gv.2014.12.05.83188

Investigation of Foot and Mouth Disease Outbreak in Egyptian Buffalo-Cows in 2012

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Abstract: Foot-and-mouth disease (FMD) is economically the most important viral disease of cloven hoofed animals. Samples from lung, tracheal and heart were exposed to Histopathological examination. Heparinized and non heparinized blood were taken from 325 mature buffalo-cows raised at Al Sharquia, Al Qalyoubiya and Al Daqhaliya Governorates of Egypt. Swabs from affected organs were submitted to routine aerobic culture and examination for *Pasteurella sp*. Samples were inoculated into BHK-21 cells and morphological examination was performed 24, 36 and 72 hrs post inoculation. The isolated virus was purified and subjected to electron microscope examination. Meanwhile the sera were tested by ELISA for FMDV. Moreover, Simple aNd Aqueous Phase hybridization-ELISA technique was performed using oligonucleotide probes targeting VP1 gene labeled with biotin. Histopathological examinations revealed sever hemorrhagic pneumonia, sever degeneration of bronchiolar epithelium and cardiac muscle degeneration with infiltration of blood and inflammatory cells. Bacteriological examination showed *P. multocida* confirmed by the standard biochemical tests. Out of 325 samples 258 positive *P. multocida* samples were screened. Virus isolation and propagation indicated the presence of FMDV confirmed by electron microscopy. ELISA showed that out of 325 samples, 285 samples (87.69%) were positive. High significant infection was among 2-6 and 4-6 years of age. The virological results were confirmed by SNAP-ELISA technique.

Key words: FMDV · Foot and Mouth Disease Virus · ELISA · EM · SNAP · Histopathology

INTRODUCTION

Foot-and-mouth disease (FMD) is economically the most important viral disease of cloven hoofed animals due to the fact that it is a debilitating and highly contagious disease. The causative agent, FMD virus (FMDV), is classified in the genus *Aphthovirus* within the family *Picornaviridae* and exists as seven immunologically distinct serotypes, namely types O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1, within which are further antigenic subtype and molecular topotype subdivisions. Rapid, sensitive and specific laboratory assays are required for FMD diagnosis to enable the appropriate control measures to be swiftly implemented to restrict spread of infection and eradicate the disease. Conventionally, FMD is diagnosed by ELISA [1]. The virus can be isolated and propagated on cell cultures and the specificity of isolated viruses confirmed by ELISA. Advances in molecular biology have resulted in the development of techniques such as the Reverse Transcription Polymerase Chain Reaction (RT- PCR) method for the detection of FMD virus genomic RNA in cell culture fluids, oesophageal-pharyngeal scrapings, epithelial or other tissues such as tonsils [2, 3]. In addition, the use of in situ RT–PCR has been applied to tissue sections and cells to determine the sites of persistence of FMD virus [4]. Recently a rapid aqueous phase (Simple aNd Aqueous Phase (SNAP)) hybridization step using biotin-labeled internal probes, which is an RT-PCR coupled with enzyme-linked immunosorbent assay was developed by Alexandersen *et al*. [5]. Unfortunately that study gave little information about applying this technique on SAT serotypes as there were no SAT-specific SNAP probes consistent with these serotypes.

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Egypt was experiencing a serious outbreak of FMD in 2012, which affected all cloven-hoofed animals, including sheep, goats, cattle, buffalo and pigs. FMD causes serious production losses and can be lethal, particularly to younger animals. An estimated 6.3 million buffalo and cattle and 7.5 million sheep and goats were at risk in Egypt [6, 7]. Although foot-and-mouth disease has circulated in the country for some years, this is an entirely new introduction of a virus strain known as SAT2 and livestock have no protective immunity against it. FMD does not pose a direct health threat to humans, but affected animals become too weak to be used to plough the soil or reap harvests, suffer significant weight loss and produce less milk. Many animals are dying from the highly contagious disease, which affects cattle, buffaloes, sheep, goats, swine and other cloven-hoofed animals and spreads through body fluids that can contaminate clothing, crates, truck beds and hay. Deaths are at high rates in young animal [8-10].

The objective of the current study was to investigate the FMD outbreak and the secondary bacteria known to accompany FMD to find the reason for the high mortality rate and to establish a rapid accurate screening system suitable for analyzing large numbers of suspected field samples. This system to be used for preliminary identification of the virus swabs, epithelial suspensions and probangs from clinical samples submitted from the field. Another aim was to focus on applying SNAP technique on FMD serotype SAT2.

MATERIALS AND METHODS

The current work was carried out during February, 2012- Juli, 2013. Collection of samples was carried out from mature buffalo-cows raised at areas of Al Sharquia, Al Qalyoubiya and Al Daqhaliya Governorates of Egypt. Animals kept in small holder farms and feed on Egyptian clover during December to May and concentrate, crop residues and rice straw during other months of the year, there is no regular system of vaccination.

Animals: The diseased animal were suffering from fever, anorexia and the appearance of vesicles on the mucous membranes of the mouth including the tongue, the dental pad, gums and lips. On the feet, lesions were most prominent at the bulbs of the heel, along the inter-digital cleft and to a lesser extent, along the coronary bands. Lesions were also present at the nares and on the muzzle, on the udder and the teats. In milking cows, there was an acute drop in milk production. Rumen and heart necropsy were frequently found especially on young animals before weaning.

Samples Collection: Blood Samples were collected from the jugular vein (heparinized and non heparinized blood); tissue samples for histopathology from lungs and heart of dead carcasses and collected mouth secretions. Samples were collected from 325 buffalo-cows. Heparnized blood was centrifuged at 3000 rpm for 5 min and the puffy coat was collected. While in non heparinized blood the blood samples were centrifuged at 2500 rpm for 5 min and sera were collected. Nasal swabs were immersed in PBS containing100 µg/ml penicillin and 100µg/ml streptomycin.

Histopathology: Routine post-mortem examination was performed on three dead Buffalo-cows and ten calves. Lungs were tested well for the presence of parasites and cysts. Lung, tracheal and heart samples were fixed in 10% phosphate buffered formalin and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 0°C for 4 h for histological examinations. Samples were embedded in paraffin, sectioned at 4–6 µm-thick slices and stained with hematoxylin and eosin (H andE).

Cultural and Biochemical Identification of *Pasteurella*: The external surface of the pneumonic lungs, liver and thoracic lymph nodes of the diseased buffaloe-cows were first seared with a heated spatula before cutting their inner surface and heart blood samples and nasal swabs were submitted for routine aerobic culture and examination for *Pasteurella sp*. Culture was attempted using standard techniques in each case. For *Pasteurella* checking, as they are the most common secondary bacteria accompanying FMD, the samples were cultured by direct inoculation on 5% goat blood agar and McConkey agar followed by incubation at 37°C for 18 h. The plates were then examined for growth and the colonies examined for *P. multocida*-like colony morphology, colour and odour. The samples were also subjected to biochemical tests to further confirmation of *Pasteurella*. The methods were performed as mentioned by Carter [11].

Cell Culture: Samples were adapted to cell culture by passaging in BHK-21 cells (obtained from Vaccine and Serum Institute, VACSERA, Egypt) using a constant amount of inoculum per passage in the absence of serum. BHK-21 cells were used for growing the virus in suspension cultures [12]. The viruses at passage 6 were further passaged twice in BHK-21 suspension cultures for bulk production and purified as described by Suryanarayana *et al*. [13]. The purified virus particles were suspended in 10 mM TrisHCl pH 7.4 containing 1.5 mM $MgCl_2$ and 10 mM NaC1 and stored at -70°C in siliconized vials.

Electron Microscopy: Some of the harvested viral suspension was used for electron microscopy at Electron Microscope Unite, Vaccine and Serum Institute (VACSERA), according to Madbouly *et al*. [14].

Virus and Sera: Reference virus and sera were kindly supplied by Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt.

Elisa Technique for Fmdv Detection: Reference virus was isolated in 96-well flat-bottomed micro-plates. BHK-21 cells were added to all the wells of the micro-plates (100 µl per well, 3×10^5 cells per ml).Then, 50 µl per well of undiluted samples or tissue suspensions and sequential 10-fold dilutions were added to eight wells per-dilution. The plates were cultured for 3 days at 37°C in a humid atmosphere containing 5% CO2, after which the supernatants of the wells were tested in an indirect double antibody sandwich ELISA for presence of antigen [15] using in-house prepared rabbit sera directed against FMD SAT-2. The ELISA was considered positive, when the optical density (OD) was greater than the mean background OD + 0.2.

Primers for Snap (Simple and Aqueous Phase) Hybridization: The primers used were targeting VP1 gene following the methodology outlined by Bastos [16] 5` CCA CGT ACT ACT TYT CTG ACC TGG A3` [16] and 5` GAA GGG CCC AGGGTT GGA CTC 3` targeting the highly conserved2A/2B junction [17]. The oligonucleotide probes used in the hybridization step were purchased already labeled with biotinat the 5`end Streptavidin-coated eight-well-microtiter strips were used for the assay (Roche Diagnostics).

Snap Hybridization and Elisa Procedure: The technique was done as reported by Alexandersen *et al*. [5]. The Biotinylated SNAP capture probes (10-20pmol) were added to the wells of a PCR microtitre plate. The PCR products were added, the plates covered, heated at 95°C and cooled to the annealing temperature (55°C). After annealing, the ELISA blocking buffer (PBS / 0.05% Tween 20 / 0.1% bovine serum albumen) was immediately added to the wells, mixed and the material transferred to a streptavidin-coated ELISA plate. After incubation at 37°C, the plate was washed and the captured product detected by anti-Digoxigenin-POD or (anti-Dig-POD) conjugate on a spectrophotometer at wavelength 405nm.

Table 1: Clinical signs of FMD infection in buffalo-cows

	FMD positive animals	
Clinical signs	FMD signs	Apparently normal
Number	270	15
Percentage	83%	4.62%
χ2 value	21.97**	

**P<0.01

Table 2: Clinical signs of FMD infection in buffalo-cows

	FMD positive animals	
Clinical signs	Number	Percentage
Fever	170	52.31
Off food	120	36.92
Lameness	170	52.31
Salivation	90	27.69
Ulcers on muzzle	190	58.46
Vesicles on feet	188	57.85
Vesicles on teat	97	29.85
More than one clinical signs	255	69.23
χ^2 value	27.74**	

**P<0.01

RESULTS AND DISCUSION

Clinical Signs: FMD is very recognizable disease from its pathognomonic lesions. Also it is very contagious disease and for this reason only 15 cases did not show any signs of the disease were positive for FMDV. Out of 325 buffalo-cows, 270 cases were clinically positive and showing signs of the disease (Table 1). Mortality rates reached 94% among calves. Table 2 shows the different symptoms observed in clinical examination. The symptoms described in this study matches with that reported by Ferris *et al*. [3].

Histopathology: Histopathology of lung tissue revealed sever hemorrhagic inflammation presented by sever congestion and perfuse inftration with inflamatory cells. and sever degeneration of bronchiolar epithelium (Fig. 1).

Moreover gross pathology show swollen heart with subcutaneous hemorrhagic spots with Tiger`s heart in young calves. Microscopic examination showed cardiac muscle degeneration with infiltration of blood and inflammatory cells which indicates hemorrhagic inflammation (Fig: 2). The results trigger the conclusion that high deaths may be attributed to pneumonia and heart failure. The results match the finding of Sandala *et al*. [18].

Bacteriological Results: The suspected colonies were Gram stained, observed and tested for the following characteristics: non-hemolytic on blood agar, acid but no

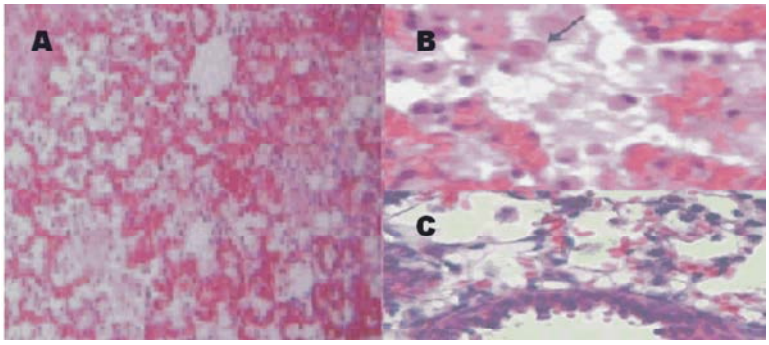

Fig. 1: Shows congestion and edema in alveoli with infiltration with inflammatory cells (A).Infiltration with macrophages and sever congestion (B). Degeneration of bronchiolar epithelium (C). (X40) stained by H andE.

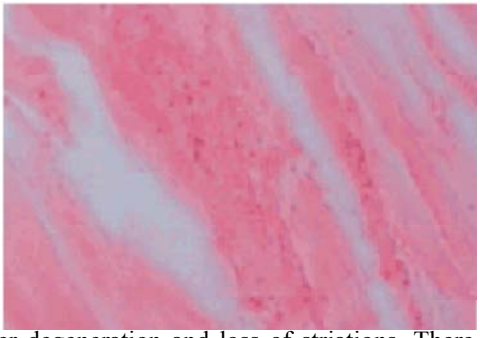

Fig. 2: Cardiac muscles undergo sever degeneration and loss of striations. There are blood and inflammatory cells infiltration.

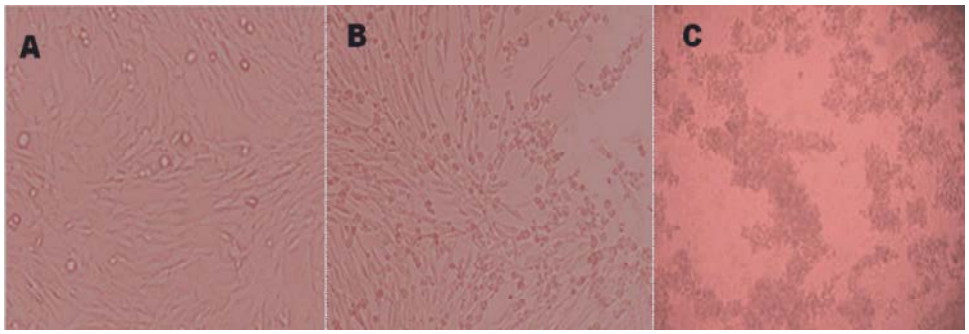

Fig. 3: Shows normal BHK-21 cells (A), 24h PI (B) and 36h PI. Cellular detachment, rounding and destruction of the cells are the most observed CPE.

gas production in triple sugar iron (TSI) agar, no motility, indole and oxidase positivity, acid but no gas from glucose and no growth on MacConkey agar. Gram-negative coccobacilli, confirmed by the standard biochemical tests above were considered *P. multocida*.

Out of 325 samples 258 positive *P. multocida* samples were screened. *P. multocida* is one of the most important causative agents of pneumonia according to Sadeghiana *et al*. [19] and Rose *et al*. [20].

Virus Isolation and Propagation: BHK-21cells showed clear cytopathic effect (CPE) after 24 hours post infection (PI) (Fig. 3B) and 36 hours post infection (Fig. 3C) with consequent induction of different degrees of degradation and cell deterioration due to progression of the virus in these cells (Fig: 3).

Electron Microscopy Findings: Examination of sodium phosphotungstate stained drops of several samples by electron microscopy, showed icosahedral capsid non enveloped virions of 25-30 nm in diameter (Fig 4). The results matches the results reported by Epstein *et al*. [21]; Schulze and Olechnowitz [22].

FMD (SAT) viruses are the three African territories serotypes named SAT1, SAT 2 and SAT 3. SAT1 and SAT2 are endemic in Sahara countries of South Africa,

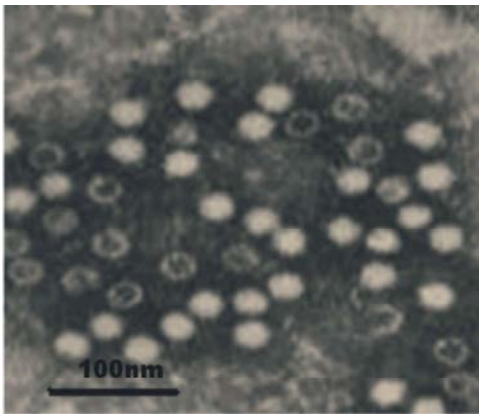

Fig. 4: Ultra-structure of FMDV small round virions approximately 30nm in diameter Detection of FMDV by ELISA and SNAP-ELISA techniques

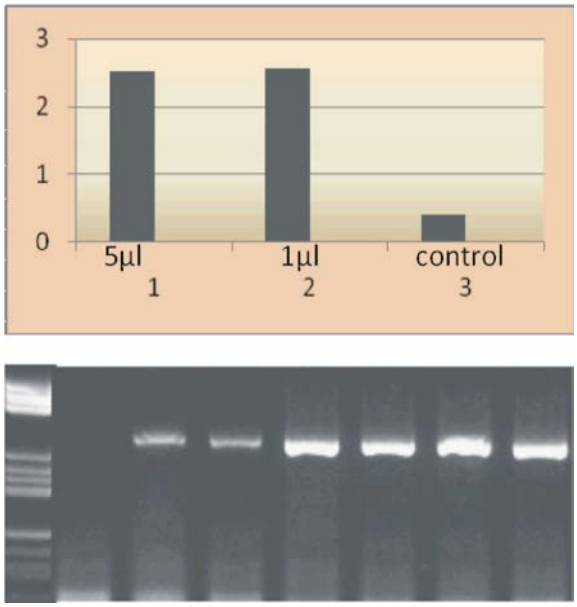

Fig. 5: SNAP probing ELISA. RT-PCR amplification. Upper photo show OD of a concentration of 5 µl, 1 µl of cDNA and control. The lower photo show RT-PCR products electrophoresed into agrose gels and stained with ethidium bromide.

Table 3: FMD infection in buffalo-cows

	FMD infected buffalo-cows		
Age (year)	Number of examined buffalo-cows	Numbers	Percentage
2-4	152	148	45.54%**
4-6	85	72	22.15%**
6-8	54	38	11.69%
8-10	34	27	8.31%
χ^2 value	21.9**		

**P<0.01

while SAT3 have been confined to a few countries in southern Africa. SAT2, the focus of this study, is the SAT serotype most often recorded in domestic animals and is widely distributed across the continent, having been identified as far west as Senegal, east as Ethiopia and south as South Africa. It is further sub-classified into 14 topotypes. In 2012, Outbreaks occurred in Egypt, the Palestinian Territories, Libya and Bahrain [23]. Ahmed *et al*. [6] conducted a genetic study of the viruses involved and found that although the bulk of the Egyptian and Palestinian isolates are closely related; those from Libya and Bahrain are of quite distinct lineages. The Bahraini virus is even of a different topotype. Furthermore, one of the samples obtained from Egypt proved to be yet another lineage, distinct from the others collected in the country during the epidemic. Kandeil *et al*. [7] note that cattle imports to Egypt from other countries in the Nile basin increased following the Egyptian revolution of 2011 due to improved political relationships between the governments involved. Virological laboratories recognize the necessity to undertake proficiency tests to establish confidence in reliability of laboratory diagnostic procedures and this is an increasing requirement of quality management systems.

ELISA showed that out of 325 samples 285 samples (87.69%) were positive (Table 3). Highly significant infection was among 2-4 and 4-6 years of age. Young age most susceptible for FMD infection than elder ones. This finding matches with the results of Remond *et al*., [12].

Alexandersen *et al*. [5] used SNAP- ELISA method described above with a highly conserved region of the FMDV genome and a single SNAP capture probe capable of binding to cDNA from FMDV serotypes except for FMDV- SAT serotypes. In the current study, VP1 region which is highly variable and is thought to play a major role in defining SAT2 from other serogroup specificity and protective immunity was used (Fig.5). SNAP technique is accurate very sensitive and rapid method of diagnosis. The SNAP-ELISA method, if lineage-specific probes are included, can give an initial indication of the virus genotype. The test is as sensitive as agarose gel electrophoresis without the hazards of ethidium bromide and is easier to use for the analysis of large numbers of samples.

In conclusion, the current study investigated FMD outbreak and established a fast and efficient SNAP hybridization step in conjunction with RT-PCR assays and ELISA. The concept of the method is demonstrated

by the detection of FMDV serotype SAT2. Furthermore, the assay can determine that the cause of high mortalities and morbidities was related to entrance of new serotype SAT2 with no previous vaccination against it, also the secondary infection by *P. multocida* caused severe pneumonia beside the cardiac infarction due to FMD SAT2 which is a main feature specially in calves forming what is called Tiger`s heart. This study recommends the use of SNAP- ELISA for large number of samples as a rapid sensitive method of diagnosis. The study also recommends vaccination against FMD SAT2 within the program of obligatory and imperative vaccination.

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