

## Serological diagnosis of FMD in sheep in Basra by ELISA test

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### Abstract

This study was performed to detect the antibodies against the virus-infection- associated antigen (VIAA) in previously diseased and healthy sheep in Basra. The test is valuable in epizootiological surveys because only infected animals with foot and mouth disease virus will give positive reaction without detection of the virus serotypes. 241 sheep sera were collected from 13 suspicious infected sheep flocks with FMD from two major areas in Basra (Abulkhaseeb and Alzubair). All these samples were examined by ELISA test to VIA antigen. It was found -by ELISA- that 71.9% of the total tested sheep sera build specific VIA antibodies against FMD virus, and that 91.7% of the clinically infected sheep gave positive result and that 66.8% of the clinically non-infected sheep were negative. The higher rate of seropositivity in both Abuelkhaseeb and AL-Zubair areas was in the age between 3.5 – 4.5 year (80%) and (81.8%) respectively. The high prevalence of seropositivity to VIA could be due to sub clinical infection or to carrier state and the disease in sheep mild and go un-noticed but important because of transmission to cattle.

**Keywords:** FMD; VIAA; ELISA; Sheep.

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### تشخيص مصلي لمرض الحمى القلاعية للاغنام في البصرة بواسطة اختبار الاليزا

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### الخلاصة

تكم أهمية هذه الدراسة في الكشف عن الأضداد المضادة للمستضد المصاحب للخمج بحمة مرض الحمى القلاعية في الاغنام المصابة سابقا بالمرض والسليمة حالياً في البصرة وذلك لما لهذا الاختبار من أهمية في عمليات المسح الوبائي حيث تظهر النتائج الموجبة في الحيوانات المصابة فقط بحمة مرض الحمى القلاعية لكن بدون تحديد النمط المصلي للحمة المسببة للخمج. تم جمع ٢٤١ عينة دم اغنام من ١٣ قطيع اغنام مشكوك بأصابتها بالمرض من منطقتين مختلفتين في محافظة البصرة (ابي الخصيب والزبير) وقد أجري اختبار الاليزا للمستضد المصاحب للخمج لجميع العينات. عند استخدام اختبار الاليزا وجد إن (٧١,٩%) من مجموع العينات المختبرة احتوت على أضداد مضادة للمستضد المصاحب للخمج بحمة مرض الحمى القلاعية وإن (٩١,٧%) من الاغنام المصابة سريريا بالمرض أعطت نتائج موجبة و (٦٦,٨%) من الاغنام غير المصابة سريريا بالمرض أعطت نتائج سالبة. وكان أعلى معدل للإصابة في كلتا المنطقتين في الفئة العمرية ما بين ٣,٥-٤,٥ سنة، (٨٠%) في ابي الخصيب و (٨١,٨%) في الزبير. أن زيادة انتشار النتائج الموجبة للمستضد المصاحب للخمج في الاغنام قد يعود لكون الاغنام حاملة للمرض فقط أو أن المرض تحت السريري لان الإصابة في الاغنام تكون معتدلة وغير ملحوظة ولكن تكمن الخطورة في امكانية نقل المرض للماشية.

## **Introduction**

Foot and mouth Disease (FMD) is the most contagious disease of cloven-hoofed animals and has a great potential for causing severe economic losses in susceptible cloven-hoofed animals, of the domestic species, cattle, pigs, sheep, goats and buffalo are susceptible to FMD, in addition many species of cloven-hoofed wildlife such as deer, antelope and wild pigs may become infected (1).

Infection of susceptible animals with FMD virus lead to the appearance of vesicles on the feet, in and around the oral cavity and on the mammary gland in females, The severity of the clinical signs varies with the strain of virus, the exposure dose, the age and breed of animal, the host species and its degree of immunity (2). There are seven serotypes of FMD virus namely O, A, C, Southern African Territories (SAT)<sub>1</sub>, SAT<sub>2</sub>, SAT<sub>3</sub>, and Asia<sub>1</sub>, infection with any serotype dose not confer immunity against other (3). A sever epidemic of food and mouth disease occurred in Iraq in 1998, affecting 3 million ruminant (about 25% of the population) and caused heavy losses in newly born animals, it is estimated to have killed about 500.000 animals; the epizootic is due to the serotype O middle East strain which was unanimously considered as extremely sever affecting not only cattle and buffalo but sheep and goat as well, which was not the case before in Iraq, all ruminant were vaccinated with two injection of vaccine and after word regular vaccination was carried out once a year, cattle were vaccinated with the trivalent vaccine (O1, A22, Asia1), while sheep and goats were vaccinated with the monovalent O1 type vaccine (4).

Clinical disease in sheep is characterized by lesions on the feet and mouth, fever, and viremia it has been reported however those up to 25% Of the infected sheep may fail to develop lesion and an additional 20% may show only one lesion (5). FMD is still prevalent in many parts of the world as emphasized by the 2001 epidemics in the European Union, southern Africa, Asia and South America (6).

Most ruminant species can harbor the virus in their pharyngeal tissues for a long period, recovered cattle or vaccinated cattle exposed to diseased animals can become healthy carriers for 3.5 years while sheep can be carriers the virus for 4 – 6 months; pigs are not carrier (7).

(8) And (9) reported the finding of third antigenic component associated with infection with foot and mouth disease virus, called virus infection associated antigen (VIA), which reacted with the sera from convalescent animals but not with sera from vaccinated animals, VIA antigen is specific for FMD but is not virus type-specific.

The antibodies against the VIA antigen were detected between the second week and 13<sup>th</sup> or 14<sup>th</sup> week after infection (10).

ELISA for antibody detection (Solid-phase competitive ELISA) is used for epidemiological survey and in the

studies of vaccines activity measurement, the test is very sensitive and specific and quick (7).

The detection of antibodies to virus infection associated antigen was done in Iraq by (11); the survey was done on Iraqi cow sera, collected from Mosul slaughter house in Mosul Government. By the Agar Gel Immuno-Diffusion, samples were examined.

The study was designed to detect a previously infected sheep with Foot and Mouth Disease (FMD), and to differentiate in vaccinated flocks between antibodies due to vaccination and antibodies due to infection.

## **Material and methods**

### **Chemicals**

The chemical materials used through the work were manufactured by the following companies; Fluka, Sigma and Serva, Pharmacia, BDH, and Difco. as the following:

Phosphate buffer saline (PBS), Na<sub>2</sub>SO<sub>4</sub>, Sephadex G-75, 13.7KD, 25KD, 45KD, 67KD, Liquid paraffin, Sodium Carbonate- Bicarbonate buffer, K<sub>2</sub>HPO<sub>4</sub>, Ammonium Sulfate, Tris, H<sub>2</sub>O<sub>2</sub>, 4-amino antipyrine, Sodium Periodat, Acetate Acetic buffer, Sodium Carbonate buffer, Sodium Borhydrate, Borate buffer, Glycerol and others.

### **Blood samples**

The investigated samples comprised 241 of Arabi sheep blood samples collected from two areas in Basra governorate during the period from March to June 2004. The first area is AL-Zubair, in which the majority samples were collected (141 samples). The AL-Zubair sheep population compromised about 80% of sheep in Basra, sheep were feed in free pasture and in contact with other species such as cow, and goats, these animals have symptoms of FMD. The samples were collected from different location in this area. The second area which is used for sample collection is Abulkhaseeb. The population of sheep in this area was less than that of the first area (100 samples) but the sheep were in contact with other species such as cow, buffalo, and goats, these animals have symptoms of FMD. The studied herds include some animals (48 cases it about 10% of sheep population) suffered from some clinical symptom of FMD as lameness, high fever and presence of some vesicles in the oral cavity and small lesion in feet, occasionally loss of hooves. High mortality rate was observed in lambs.

The samples were collected about 3 to 4 weeks after the onset of the clinical signs of FMD and there are no symptoms at the time of collection.

### **The characteristic of study population**

The age of the studied group ranged from more than 2.5 years to 5.5 years.

Regarding the geographical distribution, the majority of the studied sheep (58.5%) in- habits AL-Zubair area, while (41.5%) were taken from Abulkhaseeb area. All the test herd of both areas has the symptom of FMD before the collection in different interval and the herds were vaccinate before the infection at least two months ago.

The major characteristics of 241 sheep groups involved in this study were shown in (Table 1).

Table 1: The characteristic of study population.

	Character	No. of tested (%)
Age (year)	2.5 – 3.5 year	146 (60.5%)
	3.5 – 4.5 year	42 (17.4%)
	4.5 – 5.5 year	53 (21.9%)
Total		241 (100%)

#### **Antigen**

Virus infection associated antigen (VIAA). Obtained from the Pan American Foot – and – Mouth Disease Center Aftosa (PAFMDC) (Brazil) as a VIA in activated antigen – Lot 35 through Dr. S. Hasso.

#### **Control Serum**

A positive control obtained from PAFMDC (Brazil) – Lot OE.

#### **Enzyme conjugate**

A rabbit anti – sheep Immunoglobulin to which horseradish peroxidase has been conjugated according to the method of (12).

#### **Immunoglobulin preparation**

The Immunoglobulin (Ig) of pooled healthy sheep sera was separated by sodium sulfate precipitation a modification of (13) and (14).

#### **Estimation of protein content**

The protein content of each enzyme extracts and rabbit anti – sheep Immunoglobulin was determined by methods of (14).

#### **The fractionation of protein extract on G-75 sephadex**

The gel chromatography was used for the isolation and purification of protein extract into molecular size according to the method of (15).

#### **Rabbit anti – sheep Ig preparation**

The anti – sheep Ig was prepared in rabbits by intramuscular injection into each hindquarter, of 0.5ml of equal volumes of sheep Ig (250 µl/ml) and liquid paraffin in the form of water in oil emulsion. This was followed by booster 10 days apart, on day 30, the animal was bled 10

days after the second booster dose and the rabbit – anti sheep Ig was precipitated as described by (13).

#### **The preparation of horseradish peroxidase *Raphaus Sativus L.***

The extraction, Purification and the activity of the enzyme was estimated according to the method of (16).

#### **ELISA technique**

The ELISA technique for measuring the antibody response was established by chaquar – board titration of the antigen, control sera and conjugate. The procedure for conducting the solid phase ELISA was essentially as described by (17).

#### **Pilot study**

This test was performed to check if the antigen dilution selected from CB – ELISA is the optimal dilution or not. The highest antigen dilution that gives an optimal density reading of 0.1 with the selected serum dilution of positive samples and reading of  $\leq 0.1$  with the serum dilution of negative samples was considered as the optimal antigen dilution (18). Hence, if the antigen dilution selected from CB – ELISA is able to achieve this target in the pilot study, it will be the optimal antigen dilution. This pilot study was performed on 137 randomly chosen samples from the samples included in this study. The serum dilution, which is selected from CB – ELISA was applied in this test.

#### **Detection of the true negative**

Negative samples found in this study were tested again at serum dilution of (1/3) in order to find out if these samples contain low antibody level that cannot be detected at higher serum dilution.

#### **Testing the reproducibility of the applied ELISA**

Randomly chosen samples which have been previously tested, were subjected to repeated tests in the same environmental condition with application of the same antigen and antibody dilution of the first test.

#### **Estimation of the negative cutoff**

The negativity cutoff was estimated according to the method of (19).

#### **Statistical analysis**

The results were analyzed by one-way ANOVA test, using a statistical package for the social sciences (SPSS) version 9.0. All data were expressed as Mean  $\pm$  Std. Error. Differences between data were compared by  $X^2$  test (20).

## Results

### Result of chaquar – board ELISA

#### Determination of optimal antigen dilution

The fluctuated, nonspecific reaction with sera was noticed at VIA antigen dilution of (1:8). While the antibody – antigen reaction become symmetric and corresponding to the increases or decrease in serum dilution at VIA antigen dilution (1:4) of VIA antigen and selected as the optimal dilution, which was applied throughout the study.

#### Determination of the optimal serum dilution

The non-specific reaction of infected animal sera with the VIA antigen were noticed at serum dilution (1:9). This dilution was selected as the optimum dilution which was applied throughout the study.

#### Determination of the optimal conjugate dilution

The non-specific reaction of rabbit anti-sheep-horseradish peroxidase conjugate with VIA antigen and infected sera were noticed at conjugate dilution (1:100). The optimal conjugate dilution (1:100) was selected as the optimal dilution which was applied throughout the study.

### Test that confirm ELISA Utility

#### Result of pilot study

(60) Samples, gave values of less than 0.12. (68) Samples gave values of more than 0.15. (9) Samples gave values between 0.12 – 0.15. The background values were also less than 0.1, which is use subtracted on blanking.

#### Detection of the Negatives

It has been found that the negative serum samples also gave negative value (below 0.12) when retested at serum dilution of 1/3.

#### Testing the reproducibility of the applied:

It has been found that almost the same corresponding optical density values of the first test were given by all samples which were subjected to repeated test, with the application of the same environmental conditions and using the same antigen dilutions.

### Detection of antibodies against VIA antigen in animal sera showing clinical signs of FMD

Antibodies against VIA antigen in animal sera have a clinical signs of FMD is shown in (Table 2). The number of the infected sheep which were recorded clinically affected by FMD virus were 48 case, only 4 (8.4%) of them gave a negative ELISA test, the other 44(91.6%) gave a seropositive ELISA test. While the other tested sheep sera

came from a sheep without a clinical signs of FMD. From these samples (193), only 129 (66.8%) gave a seropositive ELISA test.

Table 2: Antibodies against VIA antigen in animal sera showing clinical signs of FMD.

Cases	No.	ELISA positive %	ELISA negative %
Cases with lameness, vesicles in mouth and hooves (FMD signs)	48	44 (91.7%)	4 (8.4%)
Cases without signs of FMD	193	129 (66.9%)	64 (33.1%)
Total	241	173 (71.8%)	68 (28.2%)

### The ELISA seropositivity in a different age group in relative to area

The distribution of VIA antibodies among the age group in relative to area was shown in (table 3). The higher rate of seropositivity in both Abuelkhaseeb and AL-Zubair areas was in the second age group (80%) and (81.8%) respectively, while the lowest rate of seropositivity in Abulkhaseeb was in the first age group (65.5%), and in AL-Zubair, the lowest rate of the seropositivity was in the third age group (70.6%).

Table 3: The ELISA Seropositivity in different age groups in relative to area.

Age group (year)	Abuelkhaseeb		AL-Zubair	
	Ex. No.	Positive %	Ex. No.	Positive %
2.5-3.5	58	38 (65.5%)	88	63 (71.5%)
3.5-4.5	20	16 (80%)	22	18 (81.8)
4.5-5.5	22	16 (72.7%)	31	22 (70.6%)
Total	100	70 (70%)	141	103 (70.04%)

$X^2 = 0.9$  DF = 2  $p < 0.05$

### The ELISA result of positive sheep sera in different age groups in relative to area

The overall ELISA result profile to VIA antibodies in relative to area is present in (table 4). In this table the highest mean of the optical density value (OD value) of Abulkhaseeb were observed in the third age group ( $0.693 \pm 0.52$ ) and the lowest mean OD value were observed in the second age group ( $0.508 \pm 0.31$ ). AL-Zubair, the highest mean of OD value was observed in the second age group ( $0.774 \pm 0.37$ ) and the lowest in the third age group ( $0.631 \pm 0.42$ ).

Table 4: The ELISA results of positive sheep sera in different age groups in relation with area.

Age group (year)	Abulkhaseeb		AL-Zubair		Total Positive No.
	Ex. No.	Mean ± STD	Ex. No.	Mean ± STD	
2.5-3.5	38	0.652 ± 0.41	63	0.621 ± 0.45	101
3.5-4.5	16	0.508 ± 0.31	18	0.774 ± 0.37	34
4.5-5.5	16	0.693 ± 0.52	22	0.546 ± 0.40	38
Total	70	0.629 ± 0.41	103	0.631 ± 0.42	173

$X^2=0.97$  DF = 2  $p<0.05$

### Discussion

At the end of 1998 and beginning of 1999 an outbreak of FMD disease occurred in Iraq affecting cows, buffaloes, sheep, and goats, the virus was isolated from cows, buffalo and sheep (5); the disease is still endemic in Basra. In this study we conducted a serological survey to detect previously FMD infected sheep in Basra using the virus infection associated antigen employing the Chaquar – Board ELISA technique. The role of area, age, and the animal history to the seropositivity to VIA were considered.

ELISA technique was used for all samples out of which 71% of the tested sheep sera gave positive results. The results were divided into strong positive, weak positive and negative samples depending on the OD value. The ELISA–VIA (3D) method was successful for this sero-epidemiological study. ELISA was successful in identification the VIA antibodies (21) and ELISA 3D method could be used as a complementary method for sero-epidemiological studies as an indirect indicator for viral activity in sheep (22).

One hundred and ninety three sheep sera from clinically healthy animals without a near history of FMD were examined, 126 (66.8%) of them were found positive to VIA in ELISA test. These results suggest that the high prevalence of seropositive sheep are mostly from the carrier state or the subclinical infection of FMD, because the disease in sheep is mild and lameness is the most obvious clinical signs of it. Most FMD cases in sheep are subclinical leading to problems in the control programs because such sheep will be diagnosed mostly only after the disease has spread or after an outbreak (23). In sheep and goat the disease is often mild and goes un-noticed but important because of the danger of transmission to cattle (3).

The disease may spread from other infected animals like cow, buffalo it contact to sheep. (24) show that high percentage of sub clinical infection of FMD occurred in sheep population kept in close contact with cattle that were affected by an endemic of the disease, while (25) mentioned that 20% of the examined non-infected sheep sera gave a seropositive result to VIA, (26) stated that the seropositivity to VIA came from some cases in which the infection was subclinical. Subclinical infection is most

important and the most difficult to confirm or refute and continue to detection of a positive animals up to four years after the last outbreak but the prevalence to disease is decrease with time due to decrease into exposure to FMD virus (27). All tested animals gave a seronegative result to VIA (21), and the sheep may be from areas free of FMD.

Sheep sera collected from both areas from sheep with previous FMD affections were tested. All were found positive to VIA with the exception of 4 samples (8.4%) found negative. These results (table 2) occur because of the animals were clinically diagnosed as a diseased with FMD. Adult sheep may develop a syndrome identical to that of cattle that it becomes a crippling disease with occasionally loss of hooves bacterial complication (3). Most of the infected animal sera gave a positive result to VIA (21) and 90% of the infected animals build specific VIA antibodies against FMD virus (28).

There was no significant effect of the area on the prevalence of seropositivity to the VIA antibodies in sheep sera in this study (Table 3), Probably due to that the sheep examined in both regions are from herds infected with foot and mouth disease or there is air born infection and it is now estimated that sufficient virus to initiate an infection can be windborne as far as 250 km (3) and the distance between both areas about 45 km, but (29) who showed that there is difference in the prevalence of the seropositivity of VIA antibodies regarding different areas due to the FMD virus was endemic in some area and not in the others areas.

There is a high seropositivity rate to VIA antigen in both areas (70%). The high rate occurred in the endemic regions or adjacent to endemic area or contact with other infected susceptible species like cows, buffalos (24). The seropositive flocks were also detected in provinces adjacent to previously infected areas or in areas along the main east to west trade route across the country and the non positive sera were detected in the region of the country considered to be free of the disease and the low level of seropositive in surveys to some herds that had high level of protection suggested that the vaccinations were effective (27).

There was a significant effect of the age of sheep on the prevalence of the seropositivity of the VIA antibodies, the high prevalence of seropositivity rate was found in the second age group (3.5-4.5 year), it may be because of the

low number of the tested sheep sera in this age group. The percentage of the positive results in animals depend on the age of the animals (28), and the age and vaccination status of the animals being sampled need to be taken into consideration (22).

The mean OD value was higher than the other age groups which revealed that the high levels of the antibody titer to the VIA in sheep sera suggesting a recent FMD infection. The presence of the high antibodies of FMD-VIA at the start of investigation period indicated that the infection was recent (30). It suggested that the risk posed by the carrier sheep to the vaccinated stock in the area should be assessed before the area is declared FMD free. Others mentioned that the peak of antibodies against the VIA were detected between the second week and the 13<sup>th</sup> or 14<sup>th</sup> weeks after infection (31).

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