

## Further Immunopathological Studies on the Female Genital System and Some Visceral Organs of Sheep and Goats Naturally Infected with Foot and Mouth Disease Virus

<sup>1</sup>S.M. Ali and <sup>2</sup>A.A. Madboli

<sup>1</sup>Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt

<sup>2</sup>Veterinary Research Branch, Department of Animal Reproduction and Artificial Insemination, National Research Center Giza, Egypt

**Abstract:** The present study was carried out on 21 ewes and 19 goats Naimy and Harry breeds aged from 2 to 4 years from different flocks in Jeddah, Kingdom of Saudia Arabia. The flocks suffered from reproductive disorders. Erosive stomatitis and profuse salivation were clinically seen. The histopathological changes revealed lymphocytic, ulcerative and hemorrhagic endometritis. In viscera, Lymphocytic enteritis, bronchointerstitial lymphocytic pneumonia, myocardial degeneration and lymphocytic myocarditis were seen. Foot and Mouth Disease Virus (FMDV) antibodies of serotype (O) in serum were detected by using ELISA in a ratio "38.09%" and "15.78%" of examined ewes and goats respectively. FMDV antigen was detected in uterine and intestinal tissue by using Avidin Biotin Complex immunohistochemistry. In conclusions, FMDV serotype (O) induced prominent histopathological changes on uterine tissue which could be affect on reproductive performance of sheep and goats.

**Key words:** ELISA • FMD • Histopathology • Immunohistochemistry • Uterus

### INTRODUCTION

Foot and Mouth Disease Virus (FMDV) is a highly contagious, clinically acute, vesicular disease of the cloven-hoofed animals, including domesticated ruminants, pigs and more than 70 wild life species [1].

FMDV is classified within the Aphthovirus genus of the family Picornaviridae. The 7 principal antigenic serotypes are the classical A, O and C types, SAT-1, SAT-2, SAT-3 and Asia [2, 3].

The etiological agent FMD virus is being a non-enveloped, positive sense RNA virus translated into a 12 structural and non-structural proteins [4, 5].

The lesion of FMD virus is characterized by the formation of vesicles in mouth, feet, teats and mammary glands. In general, sheep and goats are less susceptible than other ruminants and the clinical signs are often mild [6].

FMD virus can usually be isolated from myocardia in areas containing necrotic myocytes that are infiltrated with mononuclear cells [2, 7].

Primary pharyngeal replication of virus in oropharyngeal fluid has been strongly suggested in goats and sheep after intranasal deposition of FMDV [8, 9]. These data do not, however, rule out the possibility that virus detected in oropharyngeal fluid may have been generated in other compartments, such as the nasal cavity or lungs. Indeed, there is limited evidence that primary replication may occur in the nasal mucosa of sheep [10]. Although the specific sites of primary infection in Small Ruminant have not been documented, it is likely that distinct phases within the upper and lower respiratory tract may occur as has been demonstrated in cattle [11].

The Titration of virus in infected ovine tonsillar tissue indicated that the tonsils may play a significant role in persistence of infection in sheep; however, the finding has not been investigated further [12]. Recently,

**Corresponding Author:** Abd El-Nasser Abd El-Wahid. Madboli, Animal Reproduction and Artificial Insemination Department, National Research Center, Dokki, Giza, Postal code 12622, Tel: +233371635, Fax: +237601877, Giza, Egypt.

FMD viral antigens have been detected in lymphoid tissue in a similar pattern to that seen in cattle in the persistent phase [13].

Liquid Phase Blocking ELISA (LPBE) developed [14, 15] used for detection of antibodies against structural protein of FMDV and determines the FMDV serotype.

Immunohistochemistry (IHC) technique described as a sensitive, specific and extremely rapid tool used for detection of FMDV antigen; there is a limited literature describing the localization of FMDV antigen by IHC in domestic species [16]. IHC in relation to In Situ Hybridization (ISH) are relatively simple and able to screen far greater quantities of tissues [17].

The aim of this field study is to describe if the FMDV can gain access to the reproductive system of infected sheep and goats or not and to describe the associated histopathological changes occurred in reproductive system and some visceral organs mean while leading to decrease the reproductive performance of infected sheep and goats.

## MATERIAL AND METHODS

**Animals and Case History:** 21 adult ewes and 19 adult goats aged from 2 to 4 years and marked by the farm serial numbers as recorded in table (1) which were exhibiting a clinical signs of erosive stomatitis, off food with profuse salivation and knee position; in addition to reproductive disorders; also had a history of no vaccination against FMDV. These cases submitted from different flocks raised in west region of Kingdom of Saudia Arabia (Jeddah governorates).

## II-Samples:

**Ii-1- Blood Samples:** Blood samples were collected from jugular veins during slaughtering in a clean non heparinized glass tubes for detection of FMDV antibodies in serum by using ELISA technique.

**Ii-2-Tissue Samples:** Post mortem examination was done then tissue samples were collected from ovary, oviduct, uterus, lung, intestine and heart were taken for histopathological and immunohistochemical examination.

## III-ELISA for FMDV Antibodies Detection and Typing:

### Iii-1-Detection of Antibodies Against FMD Non-structural Protein (FMD-3ABC ELISA):

FMD-3ABC bo-ov was provided by IDEXX Laboratories, Netherlands and manufacturerd by IDEXX Lieberfeld-bern Switzerland. The test detects antibodies against non-structural proteins of FMD and was performed as described by the manufacturer's guide and according to the following calculation formula:

$$\text{Value \%} = \frac{\text{O.D samples} - \text{O.D negative}}{\text{O.D positive} - \text{O.D negative}} \times 100$$

O.D: Optical Density

**Negative:** Negative control (O.D = 0.064 =0 %)

**Positive:** Positive control (O.D = 1.220 =100%)

**III-2-Detection of antibodies against FMD structural protein (Liquid phases blocking ELISA):** The Liquid phases blocking ELISA (LPBE) kit for seven FMDV serotypes A, O, C, SAT1, SAT2, SAT3 and Asia1,

Table 1: Score of the severity and localization of the immunohistochemical findings in natural FMDV infection in comparison to FMD-3ABC ELISA and LPBE results.

Species	Case No.	FMD-3ABC ELISA and LPBE Results	Uterus	Intestine
Sheep	42	++ (O) <sup>a</sup>	++	+++
Sheep	47	+++ (O)	++	+++
Sheep	49	+ (O)	+	+
Sheep	53	++ (O)	++	++
Sheep	55	+ (O)	+	++
Sheep	56	+ (O)	+	+
Sheep	92	+++ (O)	++	+++
Sheep	106	+++ (O)	++	+++
Goats	45	++ (O)	++	++
Goats	90	+ (O)	++	+++
Goats	93	+++ (O)	++	+++

Immunohistochemistry scoring: + mild ++ moderate +++ intense.

Case No. according to farm records.

<sup>a</sup>Foot and Mouth Disease Virus Serotype (O).

obtained from the institute for animal health (IAH), Pirbright laboratory, U.K. The LPBE technique for the detection of FMDV antibodies in serum was described by Hamblin *et al.* [14, 15]. The test is based upon specific blocking of the FMDV antigen in liquid phase by antibodies in the test serum sample. Rabbit antiserum specific for the different serotypes of FMDV are passively adsorbed to polystyrene microwells. After the test serum was allowed to react with the specific FMDV antigen, the test serum/antigen mixture was then transferred to an ELISA plate coated with FMDV trapping antibodies (guinea pig antiserum to the 7 FMDV serotypes). The presence of antibodies to FMDV in the serum sample will result in the formation of immune complexes and consequently reduce the amount of free antigen trapped by the immobilized rabbit antiserum. In turn, fewer guinea pig anti-FMDV detecting antibodies will react in the next incubation step. After the addition of enzyme-labelled horse radish peroxidase (HRP) anti-guinea pig immunoglobulin (Ig) conjugate and substrate/chromogen solution a reduction in color development will be observed when compared to controls containing free antigen only and the calculation was performed according to this calculation formula:

$$\text{Percentage inhibition (PI)} = \frac{100 - (\text{OD of test serum well}) * 100}{(\text{Mean OD of antigen control})}$$

**Positive Sample:** If the OD is equal or less than 50 (i.e.  $PI \geq 50$ ).

**Negative Sample:** If the OD is greater than this value (i.e.  $PI < 50$ ).

**IV-Histopathology:** Tissue samples as ovary, oviduct, uterus, lung, intestine and heart were fixed in 10% neutral buffer formalin for 24 to 48 h. routinely processed, embedded in paraffin wax and sectioned at (5  $\mu$ m) and stained with hematoxyline and eosin (H&E) for histopathological examination [18].

**V-Immunohistochemistry:** The tissue samples collected for immunohistochemistry examination; were transformed to alcohol 70% after 24 hr. to protect the antigenicity of FMDV from formalin fixative. A commercial streptavidin/biotin immunoperoxidase kit ((EconoTek Horse radish peroxidase (HRP). Diaminobenzidine stain (DAB) Anti-Polyvalent. Scy Tek lab)) with species specificity anti-rabbit, anti-rat, anti-genia pig was used.

All procedures were done according to Haines and Clark [19] and performed according to the instruction manual. Tissue sections were treated with Proteinase K "0.1%" and incubated with Genia pig anti foot and mouse disease antibody imported from IDEXX Laboratories; Netherlands at a dilution of 1/100. diaminobenzidine chromogen substrate system was applied for color reaction. FMDV positively infected goat intestine tissues, previously confirmed with PCR, were used as positive controls. Intestine sections from sheep free from FMDV infection were used as negative control.

## RESULTS

**ELISA Results:** The results in table (1) showed that the FMD-3ABC ELISA percentage of infection in sheep more than in goats, were in sheep 8 positive cases from a total number 21 examined cases "38.09%" on the other hand in goats 3 positive cases from a total number 19 examined cases "15.78%" the positive samples using FMD-3ABC ELISA were examined to ensure the positiveness and serotyping for FMD using LPBE, the samples were positive for FMDV serotype (O). Table 1 showing a great similarity between ELISA results and the intensity of FMDV antigen persistence in different examined cases; also table (1) exhibiting that the intensity of FMDV antigen in uterus less than in intestine; were tissue localization of immunolabeling indicating the viral presence; also that was a complementary to histopathological findings.

### Immunohistochemistry Findings and FMD-3ABC ELISA and LPBE Results

#### II-histopathological Findings:

**Ii-1-macroscopical Findings and Clinical Signs:** Examined cases exhibited excess salivation, fever, depression, anorexia, serous nasal discharge and lameness with the formation of vesicles in the mouth and at the junction between hoof wall and skin. The vesicles progress to erosions and also may be found in nostrils, muzzle and teats.

#### II-2-Microscopical Findings:

**Ovary:** Severe congestion in most of blood vessels of ovarian tissue accompanied with degeneration and necrosis in the theca interna and zona granulosa cells of the mature follicles as in case No. 92, 106 in ewes and case No. 93 in goats (Fig. 1).

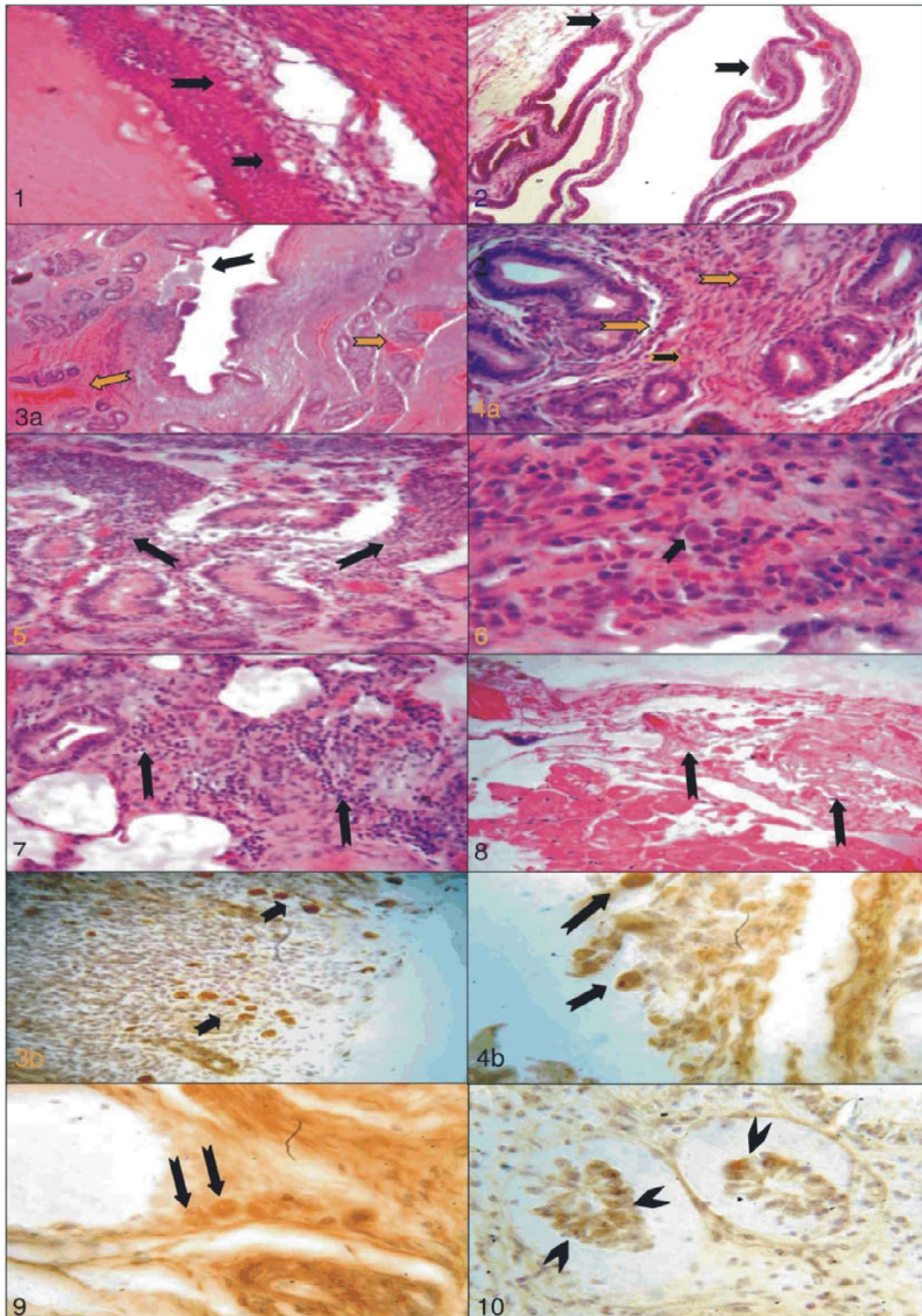


Fig. 1: Ovary; ewe, case No. 92. Showing degeneration of the cells in theca and granulosa cell layers of the mature follicles (black arrows). H&E X 200.

Fig. 2: Oviduct; ewe, case No. 106. Showing hyperplasia in the lining epithelium of the oviductal villi (black arrows). H&E X 100.

- Fig. 3a: Uterus; ewe, case No. 47. Showing ulcerative endometritis characterized by partial desquamation of the lining epithelium of endometrium with diffuse infiltration of the inflammatory cells in the lamina propria submucosa (black arrow) accompanied with extravasations of blood in the peri glandular areas of endometrium (yellow arrows) H&E X 100.
- Fig. 4a: Uterus; goat, case No. 93. Exhibiting lymphocytic endometritis characterized by moderate periglandular infiltration of mononuclear inflammatory cells mainly lymphocytes (yellow arrows) accompanied with mild periglandular hemorrhages (black arrow) H&E X 200.
- Fig. 5: Intestine; goat, case No. 45. showing ulcerative lymphocytic enteritis which characterized by complete desquamation of the lining epithelium of intestinal villi accompanied with massive diffuse infiltration of inflammatory cells mainly lymphocytes along the whole length of the intestinal tissue core of the intestinal villi H&E X 200.
- Fig. 6: Intestine; ewe, case No. 92. Showing multinucleated giant cells (black arrow) in the core of the intestinal villi which is heavy infiltrated with lymphocytes H&E X 1000.
- Fig. 7: Lung; ewe, case No. 106. Showing lymphocytic pneumonia characterized by interstitial and peri bronchial multifocal infiltration of mononuclear inflammatory cells mainly lymphocytes (black arrows) H&E X 400.
- Fig. 8: Heart; goat, case No. 93. Showing severe degeneration and necrosis of the cardiac myocytes (black arrows) H&E X 200.
- Fig. 3b: Uterus; ewe, case No. 47. Showing intra cytoplasmic positive immunoreactive stained FMDV antigen impacted in the infiltrated macrophages with pyknotic nucleus in the lamina propria submucosa (black arrow) ABC technique, Myer's hematoxyline counter stain. DAB x 1000.
- Fig. 4b: Uterus; goat, case No. 93. Showing golden brown positive immunoreactive stained FMDV antigen in the sub mucosal layer of completely ulcerated endometrium (black arrows) ABC technique, Myer's hematoxyline counter stain. DAB x 200.
- Fig. 9: Uterus; ewe, case No. 92. Showing intra cytoplasmic golden brown positive immunoreactive stained FMDV antigen impacted in the infiltrated macrophages in the lamina propria submucosa of endometrium. (black arrow) ABC technique, Myer's hematoxyline counter stain. DAB x 1000.
- Fig. 10: Intestine; ewe, case No. 106. Showing intra cytoplasmic and intra nuclear golden brown positive immunoreactive stained FMDV antigen in cytoplasm of the degenerated and necrosed intestinal gland cells (black arrow heads) ABC technique, Myer's hematoxyline counter stain. DAB x 400.

**Oviduct:** The main histopathological changes are hyperplasia in different area in the lining epithelium of the oviductal villi accompanied with congestion in some of blood vessels as in case No. 92, 106 in ewes and case No. 93 in goats (Fig. 2).

**Uterus:** The main histopathological changes in uterus were ulcerative endometritis which is characterized by partial desquamation of the lining epithelium of endometrium with extravasations of blood in the periglandular areas case No. 47, 92 and 106 in ewes and case No. 45 & 93 in goats (Fig. 3a) accompanied with diffuse periglandular infiltration of the inflammatory cells in the lamina propria submucosa of endometrium, in other areas the uterine glands were showing intra luminal infiltration of mononuclear inflammatory cells mainly lymphocytes case No. 47, 92 and 106 in ewes and case No. 45 & 93 in goats (Fig. 4a).

**Intestine:** The main histopathological findings in intestine of infected ewes and goats were severe ulcerative lymphocytic enteritis which is characterized by different degrees of desquamation of the epithelial lining of intestinal villi ranged from desquamation of the apical portion of villi to complete desquamation accompanied with severe diffuse infiltration of mononuclear inflammatory cells mainly lymphocytes in the core of intestinal villi and also in between intestinal glands as in case No. 92 and 106 in ewes and case No. 45 & 93 in goats (Fig. 5) in addition to presence of the multinucleated giant cells in the core of intestinal villi as in case No. 92 (Fig. 6).

**Lung:** Lung of infected ewes and goats were showing lymphocytic pneumonia which is characterized by interstitial and peribronchial multifocal infiltration of mononuclear inflammatory cells mainly lymphocytes

accompanied with severe degeneration and necrosis of the lining epithelium of bronchioles as in case No. 47, 92 and 106 in ewes and case No. 45 & 93 in goats (Fig. 7).

**Heart:** Heart of infected ewes and goats were showing severe degeneration and necrosis of the cardiac myocytes as in case No. 92 in ewes and case No. 93 in goats other areas were showing moderate infiltration of inflammatory cells mainly lymphocytes in between cardiac muscle bundles (Fig. 8).

**III-Immunohistochemical Findings:** Tissue localization of positive immunoreactive antigen was indicating viral presence that was complementary to histopathological findings.

**Uterus:** Uterus of infected ewes and goat was showing intra cytoplasmic golden brown moderately positive immunoreactive stained FMDV antigen impacted in the infiltrated macrophages in the lamina propria submucosa of endometrium. Nuclei of some macrophages were normal and others pyknotic. Some of these positively immunoreactive macrophages were present in areas of completely ulcerated endometrium as in case No. 47 in ewes and case No. 93 in goats (Fig. 3b, 4b) other macrophages present in the periglandular area of endometrium as in case No. 106 in ewes (Fig. 9).

**Intestine:** Moderately positive immunoreactive stained antigen was found intra cytoplasmic and intra nuclear in the degenerated and necrosis lining epithelium of the intestinal glands as in case No. 106 (Fig. 10).

## DISCUSSION

FMD is a severe, clinically acute, vesicular disease of cloven-hoofed animals including domesticated ruminants, pigs and more than 70 wildlife species [7].

OIE and WHO were classified FMD as a list A disease that means FMD has the potentiality for extensive spread between countries and can cause severe economic impact. The clinical signs of FMD in sheep and goats are often mild; so the clinical diagnosis of disease is sometimes difficult [17, 20-23]. Moreover, certain strains of the virus may be of low virulence for some species [24].

In this study we used the non-structural protein (NSP) commercially available ELISA kit which identify seropositive animals for any of the seven serotypes of FMD virus in a single test as reported by

Broonsvoorts *et al.* [25] and so, in our study revealed 8 positive sheep out of 21 and 3 positive goats out of 15 using commercial FMD-3ABC ELISA kit; also LPBE ELISA test used for confirmation, in which detection of antibodies to structural protein confirms current or previous infections with FMD virus in unvaccinated animals as mentioned by Periolo *et al.* [26], so LPBE in this study confirmed the detection of FMD antibodies and serotyping of FMD according to Hamblin *et al.* [14, 15] and Terzic *et al.* [27] which showed the infection of 8 sheep and 3 goat with FMD virus serotype (O).

The variety of susceptibility of FMD infection in sheep and goat is related to breeds of animals and strain of virus; the serotype (O) FMD virus has only once been isolated from a species other than pigs [28].

Our study revealed that the susceptibility of sheep to FMDV infection "38.09%" are more than in goats "15.78%" that may explained by the fact that; in adult sheep, recovery from FMD uncomplicated by secondary pathogens, is usually rapid, but the virus will persist in the tonsillar tissue for up to nine weeks in sheep and for shorter period in goats, that leads to the positivity of sheep more than in goats [29].

The histopathological finding in uterus showing ulcerative and lymphocytic endometritis; in intestine lymphocytic enteritis was also seen. In lung; Lymphocytic Interstitial bronchopneumonia was observed. These findings can be explained by the receptor distribution and antigenic structure of FMDV which contains four structural viral proteins VP1, VP2, VP3 and VP4 [4]. VP1 contains the highly conserved sequence, arginine-glycine-aspartic acid (RGD), which has been shown to be a recognition sequences for cell surface receptors enable binding and subsequent cell adhesion, virus internalization into acidic endosomes, signaling, cell migration and thrombosis [30-33]. The major receptors for FMDV in cell culture are  $\alpha\text{v}\beta 1$ ,  $\alpha\text{v}\beta 3$ ,  $\alpha\text{v}\beta 6$  and  $\alpha\text{v}\beta 8$  [34-40],  $\alpha\text{v}\beta 6$  is expressed constitutively on the surface of epithelial cells meanwhile on the endothelial lining of blood capillary at sites which are normally targeted by FMDV; these knowledge explain the presence of virus in the endometrium and intestine which confirmed by the Immunohistochemical technique in uterus and intestine.

Heart of infected sheep and goats showing severe degeneration and necrosis of the cardiac myocytes accompanied with lymphocytic myocarditis, these findings can be clearly explained by Gulbahar *et al.* [1] who reported that; FMDV-infected lambs showed lymphocytic myocarditis which considered as a significant expression to stimulate the cardiac myocytes

to release Inducible nitric oxide synthetase enzyme (iNOS) and in turn the nitric oxide (NO) abnormally increased [41, 42]. High concentration of (NO) leads to the formation of toxic products like dinitrogen trioxide and peroxynitrite, which induce cell death, if not by apoptosis, then by necrosis [43].

The immunohistochemical findings in this study revealed the detection of FMDV antigen in the endometrial tissue and also the histopathological changes found in uterus that means there are a great direct effect on the reproductive performance of infected sheep and goats, these finding come in accordance with some authors who reported that; There is a substantial evidence upon infection of gravid ewes, abortion may occur [44]. Some reports have suggested more severe clinical disease in periparturient ewes compared with non-gravid cases [45]. The only published experimental studies examining FMD during pregnancy showed that FMDV can cross the placenta at 45, 75 and 90 days of gestation resulting in foetal death [46, 47]. More over the higher incidence of trans-placental transmission may occur in dams infected at earlier stages of gestation [48].

## CONCLUSION

This study could be concluded that; FMDV serotype (O) can be gain access to the reproductive system of ewes and goats and induced prominent histopathological changes on uterine tissue which could be affect on the reproductive performance of sheep and goats.

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