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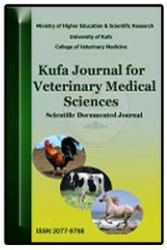

Pathological and molecular study of mycotic abortion in ewes

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Abstract:

The aims of the present study is to isolate the fungal species associated with ewe abortion and to examine the histopathological changes in the placenta associated with fungal isolates .In addition to study the genetic information of TNF alpha gene, intensity and score of TNF-alpha protein in aborted and normal placenta by PCR and IHC respectivly.

Frozen placental tissue and paraffin embedded tissue belong to aborted and normal groups were used for assessment of TNF alpha gene by studying the genetic information of TNF-alpha gene in aborted and normal placental sample by PCR .immmunohistochemical study was carried out to determine TNF protein in the placental sample. The same frozen placenta tissue samples were also used for DNA extraction to detect TNF alpha gene in the two groups.

A fragment of (238 bp) of the TNF alpha gene was amplified by PCR using primers ,the sequence of sense and antisense primer for TNF alpha gene was:
GAA TAC CTG GAC TAT GCC GA,
CC TCA CTT CCC TAC ATC CCT (bp 238) Gene Bank: X56756.

The study showed that 10 fungal species were isolated from aborted placenta with high percentage of these isolates was recorded in March ,.The fungal isolates were variable according to region of sample collecting ,high percentage in the AL-Manathera(36%),followed by Al-Kuzweenia (34%) and Al-Shabaka(30%).

The fungal isolates include *Coccidioides immitis* (16%). *Asperigllus.fumigitus* (14%), *Trichophyton, Rhodetella rubra,* and *Aspergillus.ochraus* ,(10%) for each one . *Candida kruzei*,(8%). *Aspergillus.flavus* and *Geotrichum* ,6%) for each one . *Prototheca..zopfi*, *Saccharomyces.cerevisia*, *Blast.capitatuss*, *Cryptococcus uniguttultus* and *Candida zeylanoides*,(4%) for each one .

This study revealed the presence of ~238bp band in addition to the ~300 ,400 and 600 bp desired fragment in PCR products that related to aborted samples compared to normal one .,this may be due to polymorphism within the TNF gene itself.

We found a significant elevated TNF alpha protein in inflammatory cell (macrophage) of aborted sheep placenta (increasing of intensity and score), while no changes could be observed for pro-inflammatory molecules in the control sample (normal delivery)

The current study expressed correlation between polymorphisms in TNF- alpha gene ,immunohistochimistry and the fungal isolated species.

The histopathological examination showed acute suppurative placentitis, necrosis of chorion villi, congestion of blood vessels with fibrin deposition in the villus stroma and inter villus space, in addition to calcification in the villus stroma and sloughing and desquamation of cytotrophoblast .numerous yeast and spheriols were detected in the placental tissue by periodic acid Schiff reaction(PAS).

Key words: mycotic abortion, TNF alpha ,PCR,.

دراسة مرضية وجزيئية لمسببات الاجهاض الفطرية في الاغنام

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الخلاصة:

تناولت هذه الدراسة عزل الفطريات المصاحبة لحالات الاجهاض في الاغنام في محافظة النجف وكذلك دراسة التغيرات المرضية في مشيمة هذه الحيوانات كما تم استعمال طريقة IHC وPCR في هذه الدراسة لتحديد كمية ومكان انتاج المحركات الخلوية الالتهابية(TNF alpha) في مشيمات الاغنام المجهضة ومشيمات الاغنام الوالدة طبيعيا.

تم استعمال النسيج المجمد الماخوذ من مشيمة الحيوانات المجهضة والوالدة طبيعيا لدراسة(TNF alpha) عن طريق معرفة المعلومات الجينية ل(TNF alpha) وذلك باستعمال تقنية (PCR) عن طريق استعمال بادات خاصة ل(TNF alpha) ذي الوزن الجزيئي (238bp) وحسب تردد القواعد الامينية التالية:

GAA TAC CTG GAC TAT GCC GA,
CC TCA CTT CCC TAC ATC CCT

كذلك تم استعمال النماذج المذكورة انفا في استخلاص الحامض النووي (DNA) لتقويم (TNF alpha) في مشيمة الحيوانات المذكورة انفا.

تم عزل 10 انواع فطرية من 50 مشيمة اغنام مجهضة (*Coccidioides spp*) بعدد 8من مجموع 50 وبنسبة (16%) و(*Aspergillus fumigate*s) بعدد 7 من مجموع 50 وبنسبة (14%) و(*Aspergillus ochraus*, *Trichophyton spp* and *Rhodotella rubra*) بعدد5 من مجموع 50 وبنسبة (10%) و(*Candida krusei*)بعدد 4 من مجموع 50 وبنسبة (8%) و(*Aspergillus flavus* and *Geotrichm*) بعدد 3 من مجموع 50 وبنسبة (6%) و(Candida *Cryptococcus uniguttulutus* , *Saccharomyces.cerevisia* *Prototheca..zopfi* zeylanoides, and Blast.capitatus) بعدد 2 من مجمو ع 50 وبنسبة (4%) .ومن خلال هذه النتائج تبين ان فطر (Coccidioides spp) هو من اخطر الفطريات المعزولة في هذه الدراسة وتم تسجيله لاول مرة في العراق.

من خلال نتائج (PCR) ل(TNF alpha) ، اظهرت هذه الدراسة وجود حزم اضافية ذات وزن جزيئي 300-400-600 فضلا عن الحزمة الاعتيادية ل(TNF alpha) ذي الوزن الجزيئي (238) . وقد لوحضت هذه التغيرات في مشيمة الحيوانات المجهضة مقارنة بالنماذج العائدة الى الحبوانات الوالدة طبيعيا ومن الممكن ان يكون هذا سببه حدوث تغيرات او تحويرات (polymorphism in TNF alpha) .اما من خلال نتائج (IHC) لبروتين (TNF) فقد تم ملاحضة زيادة ملحوضة في بروتين (TNF) في الخلايا الالتهابية (البلعمية) العائدة الى مشيمة الحيوانات المجهضة ،في حين لاتوجد هنالك اي تغيرات ممكن ملاحضتها لهذا البروتين في مشيمة الاغنام الوالدة طبيعيا .وقد تم ملاحظة وجود علاقة مترابطة بين نتائج (PCR) و(IHC) ففي حالت وجود التغيرات (polymorphism) ،تكون هنالك زيادة ملحوظة في البروتين المنتج . تبين وجود ارتباط بين ضراوة الفطر المسبب للاجهاض وبين التغيرات الخاصة ل(TNF alpha). اما بالنسبة الى التغيرات المرضية في مشيمة الاغنام المجهضة فقد شملت وجود التهاب قيحي مشيمي متموت فضلا عن احتقان الاوعية الدموية وترسب الكالسيوم في انسجة المشيمة ، كذلك وجود انسلاخ لخلايا (syncytotrophoblast) في انسجة مشيمة الحيوانات الجهضة .اما التغيرات المرضية في اجنة الحيوانات المجهضة فقد تم ملاحضة التهاب رئوي قيحي، والتهاب كبدي تموتي والتهاب الطحال الليفي التموتي كذلك فمن اهم التغيرات المرضية التي تمت ملاحظتها هي وجود(Spheriole) في المشيمة واجنة الحيوانات المجهضة المصابة بفطر (Coccidioides spp).

Introduction:

Abortion meanS interruption of pregnancy during the final two months that lead to very important health and economic problem in farm animals worldwide.(1) Hill *et al*.,(2) explained that recurrent spontaneous abortion has been related to a high degree of maternal immunity during pregnancy.

An interaction between the conceptus and immune system is important during pregnancy(2).Maternal infections during pregnancy have been associated with a variety of gestational complications, including pregnancy loss (particularly in the second trimester, preterm birth, and poor neurological outcome in the child.(3), also (4) and.,(5) explained that the inflammatory processes induced by host defense to infection or by immune disorders independent of infection are a major challenge to successful pregnancy and are linked to fetal growth restriction .

The effects of infection on pregnancy and fetal development are mediated in large part by proinflammatory cytokines(6),these cytokines have direct access to the placenta via maternal blood and signaling may be propagated across the placental barrier through stimulation of inflammation within the placenta (7). TNF-α is known to have detrimental effects on the placenta via its cytotoxic effects, which are mediated largely through TNF receptor 1 (TNFR1) through its intracellular death domain, which activates the caspase apoptosis pathway ,also TNF alpha ,can act in gestational tissues, are target uterine endothelial cells and elicit vascular injury and placental ischemia and cause fetal injury leading to placental and fetal damage (8).

However, there are different etiology that induce abortion such as stress factors, pharmaceuticals ,nutritional deficiency and toxic plantS ,in addition to biological agents, bacteria, viruses, parasites and mycotic agents(1).

Fungi can produced reproductive failure in animals either by direct infection of the genital system or by producing toxic metabolites(mycotoxin) which are subsequently ingested and absorbed and induced mycotic abortion which is the most important subsequence of fungal infection of the genital tract. mycotic infection has worldwide distribution and may cause placentitis and abortion in almost all domestic animals. Different fungi have been isolated from aborted fetuses, belonging to the species *Aspergillus fumigatus* and *Aspergillus nidulans, Absidia corymbifera, Mortierella wolfii, Rhizopus spp., Mucor spp., and Rhizomucor* (9).

In Iraq ,there are little information about the mycotic abortion in sheep and the molecular changes in TNF-alpha gene in the aborted placenta of sheep as well as ,there is no report about *Coccidioides* spp , therefore the present study aimed to determine the fungal spp associated with abortion in sheep and the pathological changes in the placentitis associated with these fungal infection as well as the molecular changes in the TNF gene associated with fungal isolates from aborted placental ewe .

Material and Methods:

Sample collection

During March to June ,2011, 50 placental samples were collected from aborted and normal delivered ewes from various region of AL-Najaf city, The samples were sent immediately under refrigeration to Lab .of Vet .Pathology, each sample was divided into three parts, one part was fixed in 12% neutral buffer formalin for histopathological study and immunohistochimistry ,other part was stored at -20C for molecular study and other part was used for fungal isolation.

Portion of aborted placenta was placed in a beaker and was washed by sterile normal saline to remove debris then by

sterile scalpel ,we cut the cotyledone and caruncles into small pieces ,then we cultured these specimen on the surface of the plate of Sabouraud dextrose agar containing chloramphenicol 0.05 gm\ml and incubated at $30C^0$ for 2-5 days ,any fungal growth was stained with lactophenol cotton blue.

Fungal isolates were identified by colonies ,microscopic characteristics ,germ tube test, Indian ink and biochemical tests ,in addition the yeast isolates were confirmed by API-yeast –identification system. Suspected diagnosis of Coccidioides spp were confirmed by RAPID PCR and RT-PCR According to Lindsley et al.,(10)

Molecular Detection of TNF alpha gene in placental tissues

DNA extraction from frozen tissues samples was performed using a commercial kit { Genomic DNA Mini Extraction Kit(tissue)} following the manufacturer's instructions. 90-100 mg of placental tissue was cut and transferred to 1.5 ml ependdorff tube.

Briefly, 100µL of thawed homogenates o f placental tissues were mixed with 400µL of GT Buffer and continued homogenization by grinding . followed by the addition of 50µL proteinase K (20mg mL-1) and the Samples were vortexed and incubated at 37°C overnight. Buffer GBT (200 µl) was added to each sample and vortexed. Samples were incubated in at 60°C for 20 min. The samples were centrifuged, transferred the supernatant to a new 1.5 ml microcenterfuge tube. two hundred µl of 100% ethanol were added to each sample and vortexed. The samples were then quickly centrifuged and the supernatant was split over two spin columns. The columns were centrifuged for two minutes at 14 rpm .The flow – through was discarded and 400 µl of buffer W1 was added to each column. The columns were centrifuged for 30 seconds at 14 rpms and the flow through was discarded. Washing Buffer (600 µl) was added to each column and centrifuged for 30 seconds rpm ,discarded the flow through ,then centrifuge for 3 minute at 14 rpms to dry the coloum matrix . The columns were placed into new sterile 1.5 ml microcentrifuge tubes and 100 µl of Elution buffer was added to each column. The columns were incubated at room temperature for 5 minutes and then centrifuged for one minute at 14 rpm, the resulting extracted DNA was stored at -20°C until amplification

Quantitation and quality assessment of DNA

Quality and purity of DNA were checked by submarine agarose gel electrophoresis using 0.8% agarose in 0.5X TBE (pH 8.0) buffer (11). Ethidium bromide (1%) was added 6µl/100ml. The wells were charged with 10µl of DNA preparations mixed with 3µl of 6X gel loading buffer dye. Electrophoresis was carried out at 5V/cm for 40 min at room temperature and then the DNA was visualized under UV transilluminator.

Amplification of TNF alpha Gene

Table 1 List of Primers for TNF alpha

References	Product length(bp)	Sequence 5'─ 3'	Name of primers (TNF-fa)
Gene Bank: X56756	238	GAA TAC CTG GAC TAT GCC GA	P1(F)QA9482
		CC TCA CTT CCC TAC ATC CCT	P2(R)QA9483

PCR of Placental tissue DNA was carried out in final reaction volume of 25 µl in thermal cycler (MyCycler, Bio-Rad, USA). Quantity and concentration of various components used

for Placental tissue DNA PCR are shown in Table(5). Steps and conditions of thermal cycling for different primer pairs in PCR are shown in Table (6).

Table (2) Quantity and concentration of various components used in PCR

1	2+KAPA2GRobust hotstart Ready Mix contains 2mM $Mgcl_2$,at 1X	12.5 ul
2	Forward Primer (10 uM)	2 ul
3	Reverse Primer (10 µM)	2 ul
4	Template DNA	3 ul

Table (3) Steps and conditions of thermal cycling for different primer pairs in PCR

Cycling conditions					
Final extension	Extension	Annealing	Denaturation	Initial denaturation	
72°C 5 min	72°C 10 min	60°C 10 sec	95°C 10 sec	95°C 10 min	
1	35 Repeated for 35 cycles			1	

Immunohistochemical analysis for the detection of TNF alpha antigen in paraffin embedded sections

paraffin- embedded sections from each specimen were cut at 5 micron, mounted on glass and dried overnight at 37˚C . The tissue sections were deparaffinized in xylene (2×10 min) and dehydrated through graded alcohol (100 % ,2× 5 min)and 95 % and 70 % for 5 min). Endogenous peroxidase activity was blocked using (1-1.5) % H2O2 for 5-10 mintus. .Tissue samples were heated to retrival antigens in citric buffer (pH 9.0) at 100 ˚C for 10 min .The sections were incubated with mouse monoclonal antibody against TNF alpha (diluted 1:50 over night at 37 C^o. Then sections were washed in buffer solution and covered with Biotinylated secondary antibody for 2 hours at 37 C^o, AB enzyme was applied for 30 min at 37 C^o .

Further processing of the sections for detection was performed using the dextran-polymer method , and diaminobenzidine (DAB; Sigma). After being washed, the sections were counterstained with Mayer's hematoxylin, washed in water,and successively immersed in graded ethanol solutions and xylene before cover slipping. All samples were processed under the same conditions. When counting the number of positive cells in the staining tissues samples, at least 10 high-power fields were chosen randomly on each section. Additionally, the number of macrophage was counted in the fields.

All immunostained sections were examined by the same two observers with a ×400 objective lens under the light microscope (Olympus Bx50;Olympus Optical Co, Ltd, Tokyo, Japan) for evaluation of TNF alpha expressions. In the abortion and normal delivery specimens ,TNF alpha expression in macrophages was evaluated by counting 1000 cells of each section. TNF alpha expression was quantitatively assessed as 0 (no stained cells), score 1 (from 1-25 positive cells), score 2 (from 26-50 positive cells), score3(from 51-75 positive cells)and score 4(from 76 and over). The intensity was scored as 0 (absent), (low), (moderate), or (high). The pattern and intensity of staining in the different cell types of placental samples was evaluated by two independent observers using a light microscope at a magnification of 200 x . The degree of staining in each placental

cell type was graduated as described by (12). The presence of lesions in the placental samples was investigated in formalin fixed tissue samples embedded in paraffin from those cases that had tested positive for mycotic abortion (tables 3.1 and 3.2)..

In cases where serial sections had been used for H&E and IHC section comparisons could be made between lesion sites and IHC labeling. Histological evaluation of the placenta was revealed occasional necrosis of endothelial cells. However, immunohistochemical labeling allowed the identification of TNF alpha antigen in all cases studied. Lesions were visible in all samples, after the likely lesion sites had been identified by the IHC labelling.

Result:

The preset study, showed the overall percentage of the fungal species was: Geotrichm 3 out of 50(6%) followed by Trichophyton spp, Rhodetella rubra and Aspergillus ochraus 5 out of 50-(10%) for each one ,Candida krusei, 4 out of 50(8%) Coccidioides immitis 8 out of 50(16%), Cryptococcus uniguttultus ,Sac.cereviiae, Pro.zopfil, Candida zeylanoides and Blast.capitatus,2 out of 50(4%) for each one ,Aspergillus flavus,3 out of 50(6%) and Aspergillus fumigitus ,7 0ut of 50(14%)(Table:5), Our result recorded that all collecting samples were positive for fungal isolates and the percentage of these isolates was different according to region of sample collection(Table:4),the higher percentage was recorded in the samples that were collected from Al-Manatheria,18 out 50,(36%) followed by AL-kuzweenia out 17(34%) and AL-Shabaka ,15 out 50(30%).

Table:4. shows number of fungal isolates from different regions during March to June 2011

Area of sample collection	Total samples	+ev fungal isolates	%
Al-mananthera	18	18	36
Al-kuzweenia	17	17	34
Al-shabaka	15	15	30

Table :5 Number of fungal spp according to area of samples collection

	Fungal spp	No of spp	%
1	*Coccidioides immitis*	8	16
2-	*Asp.fumigitus*	7	14
3	*Trichophyton spp*	5	10
4	*Rhodetella rubra*	5	10
5	*Asp.ochraus*	5	10
6	*Candida kruzei*	4	8
7	*Asp.flavus*	3	6
8	*Geotrichum spp*	3	6
9	*Prototheca..zopfi*	2	4
10	*Saccharomyces.cerevisia*	2	4
11	*Blast.capitatuss*	2	4
12	*Cryptococcus uniguttultus*	2	4
13	*Candida zeylanoides*	2	4
total		50	100

We demonstrated that *Aspergillus spp* form 30% of fungal isolates , In addition, *candida krusei* and *Candida zeylanoides* form 6 out of 50 (12%) of fungal isolates ,. The present study showed that *Cryptococcus.uniguttulutus* formed the lower fungal isolates (4%) , The present study also reported that *Rhodotella rubra* was isolated from 5 cases of abortion,(10%) , also we reported that *Coccidioides immitis* form high fungal (16%) isolates, associated with abortion of the ewe.

The morphological aspect of the colonies on Sabouraud agar, the microscopic aspect of the hyphae, sporangia and sporangiospores and the execution of the PCR confirmed the diagnosis of *Coccidioides immitis* in 8 cases of aborted ewes. However ,the isolation of *Coccidioides immitis* during the course of the study occur during spring season, Six out of 8 *coccidioides immites* isolates were identified in the placental samples, that were collected from aborted ewes in Shabaka ,these area characterized by dry soil.

The current study was considered as the first study that reported *Coccidioides immites* in Iraq.

Molecular examination of TNF alpha gene

The result of Gel electrophoresis of PCR products revealed the presence of -238bp band, in addition to the -300,400 and 600bp desired fragment(fig:1).

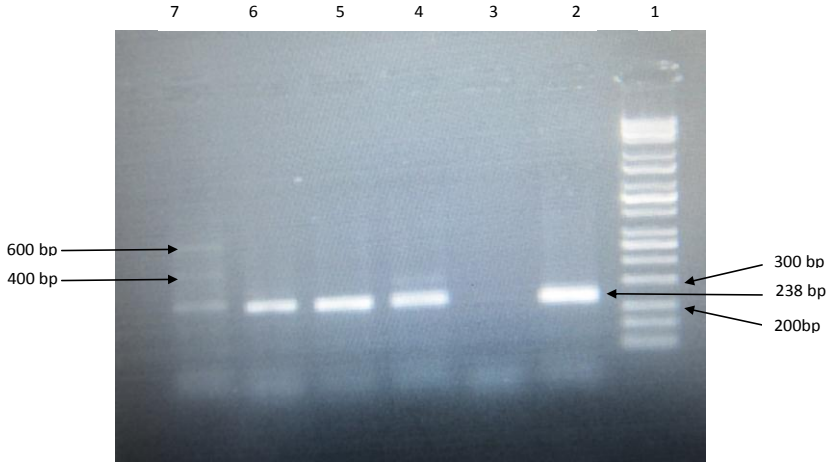
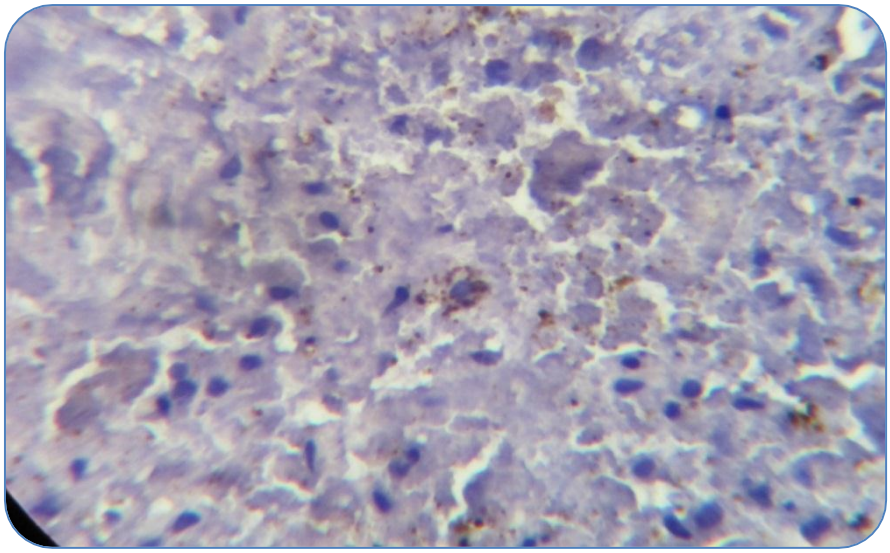


FIG (1)-Amplified DNA of TNF –alpha gene in ewes Lane-1:Ladder universal ladder kit 100-10000 bp size Lane-2:TNF-alpha gene amplified from normally delivered ewe at 238 bp Lane 3.controlLane4&5:Amplifid TNF-alpha gene in aborted ewes at the level 238 bpLane 6:Amplification of TNF-gene 2 bands 238 &300 bpLane 7: Amplification of TNF-gene 3 band 238,400 &600 bp.

Results of immunohistochemistry

Immunopositivity of TNF alpha protein was high among the necrotic cells in the placenta of aborted ewes (fig 3). Whereas, in the placental tissues from normal delivered ewes,TNF alpha immunopositive cells were markedly few (fig 2) ,and there was significant differences between the aborted animals and the delivered animals. Healthy



FIG(2):Placenta from normal delivery ewes showing TNF-alpha expression (cytoplasmic staining) of Macrophage .Score 1, Moderate Intensity.(IHC).Stained by DAB-chromogen (Brown) and counter by Hematoxyline.400X.

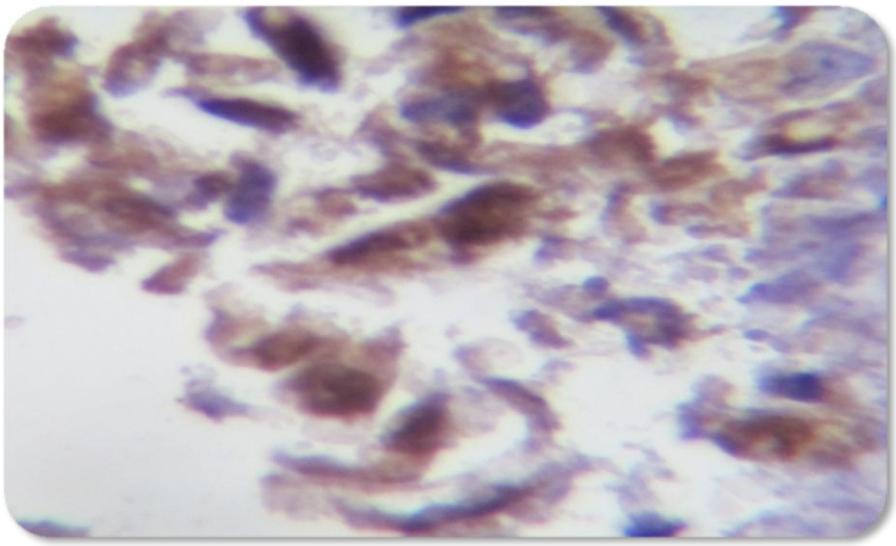


Fig (3):Placenta from aborted sheep showing TNF-alpha expression (cytoplasmic staining) of Macrophage .Score 3,high Intensity.(IHC).Stained by DAB-chromogen (Brown) and counter by Hematoxyline.400X. v

We reported that, there is a relationship between the presence of multiple bands of TNF alpha gene which were recorded by PCR, with the intensity of TNF-alpha protein that recorded by immunohistochemistry(fig 3) and the fungal species isolates.(table 6)

	Causes	NO. Abortion In the ewe	PCR Results		Immunohistochemestry Results	
			Polymorphism	No. of bind Polymorph	Score	Intensity
1	*Candida zeylanoid*e .	2	-no polymorph	No additional bind	2	Moderate
2	*Coccidioid*	1	polymorphed	3	4	High
.3	*Coccidioido*	1	=	2	4	=
4	*Rhodotella*	2	-	-	1	=
5	Blast.capitatus	2	=	-	1	Low
6	*Prototheca..zopfiz*	2	=	-	2	Moderate
7	*Rhodotella*	2	=	-	1	=
8	*. Aspergillus flavu*	1	polymorph	2	3	Hight
9	*Rhodotella rubra*	2	-	-	1	=
10	Blast.capitatus	2	=-	-	1	Low
11	es*Coccidioid*	1	polymorph	3	4	High
12	Saccromyses	2	-	-	1	Low
13	*Aspergillus ochrau*.	1	polymorph	3	3	High
14	*Prototheca..zopfiz*	2	-	-	1	Low
15	*Rhodotella rubra*	2	=	=	2	Moderate
16	Ochrasious ..Asp	1	polymorph	2	4	High
17	Coccidioido	1	=	3	4	=
18	*Aspergillus ochra*sus.	1	=	2	3	=
19	*Aspergillus ochrasus*	1	=	3	4	=
20	*Coccidioi*des	1	=	3	3	=
21	*trichophitum*	2	no	no	2	Low
22	*Aspergillus ochra*sus.	2	polymorph	2	2	Moderate
23	*Aspergillus flavu*	1	polymorph	2	3	High
24	*Aspergillus fumigitu*	1	=	2	4	=
25	*Coccidioido*	1	=	3	4	=

In addition, we recorded the presence of severe pathological lesions in the placenta that associated with high intensity of TNF-alpha ,which were recorded by immunohistochemistry , However, ,we found high score and intensity of TNF-alpha protein that were recorded by immunohistochemistry in the aborted

placenta in which *Aspergillus .spp .Coccidioidomyes spp.Candida sp*p and *Cryptococcus spp* were isolated but low score of TNF-alpha protein by IHC ,which were reported in the placental tissue in which other fungal spp were isolated .(table 6)

The present study is the first report about the TNF gene polymorphism in placenta of aborted ewes in Iraq .

Pathological changes

Macroscopic findings of the aborted placenta showed congestion with necrotic areas.

Histopathological examination

The main lesions characterized by necrosis of chorionic villi with congestion ,as well as necrosis and sloughing of trophoblastic cells of the chorionic villi with neutrophils infiltration in the villous stroma as well as congestion of blood vessels with neutrophils in their lumen ,and in the necrotic area of the choronic villi ,necrosis of trophoblasts with fibrin deposition with inflammatory cells infiltration in the intervillous space were seen ,in addition,congested blood vessels with neutrophils in their lumen in the connective tissue plate (Fig:4)

Histopathological section also revealed edema and vacuolation of cytotrophoblasts (Fig:5) in addition,yeast that stain pinkish color with PAS was seen in the necrotic tissue (Fig:6) as well as in necrotic plate connective tissues(Fig:7).

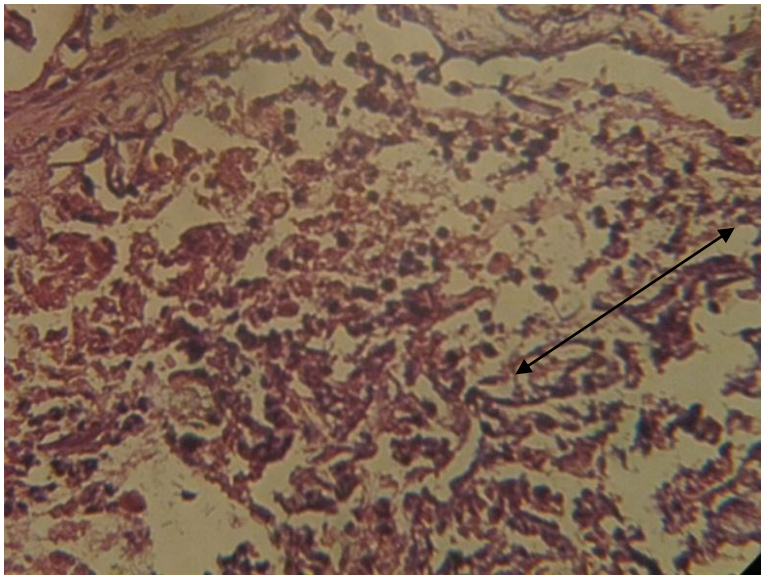


Fig:4.Histopathological section in the placenta of aborted sheep shows severe necrosis of cotyledon ,congestion of blood vessels with neutrophils infiltration in the necrotic area (H&Estain 40X)

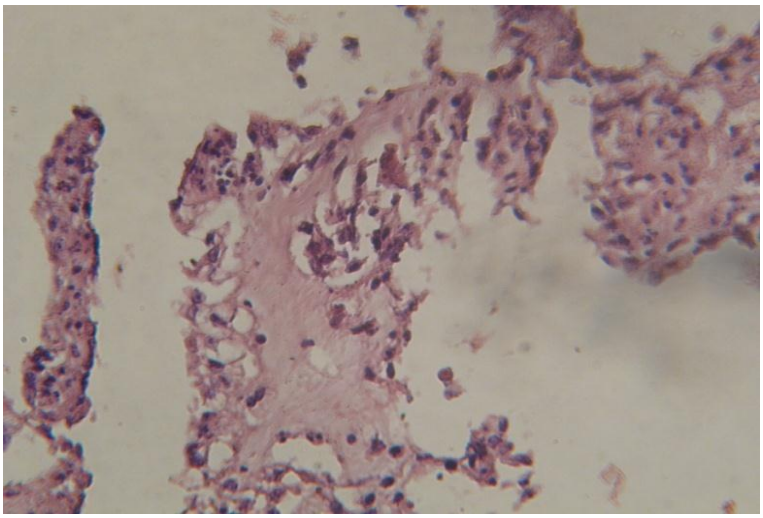


Fig:5. Histopathological section in the placenta of aborted sheep shows edema. congestion of blood vessels with neutrophils in their lumen and vacuolation of trophoblasts (H&E stain 40X)

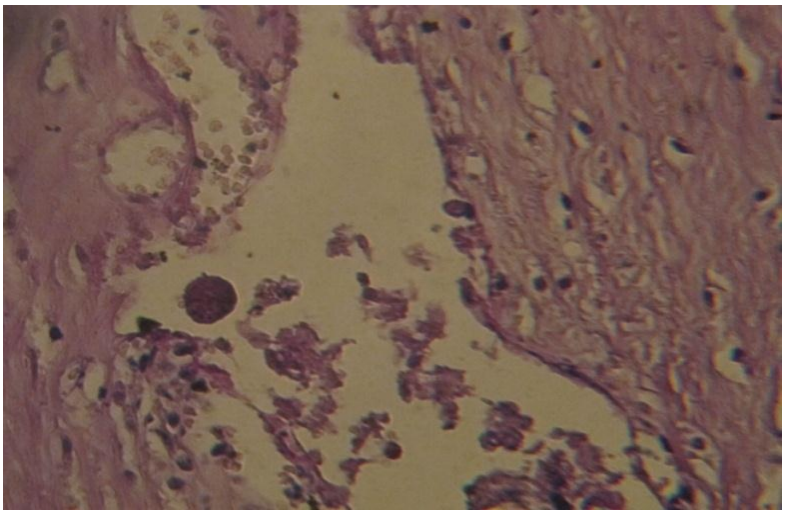


Fig:6 Histopathological section in the placenta of aborted sheep shows congestion of blood vessels with neutrophils in their lumen yeast in the necrotic debris (PAS stain 40X)

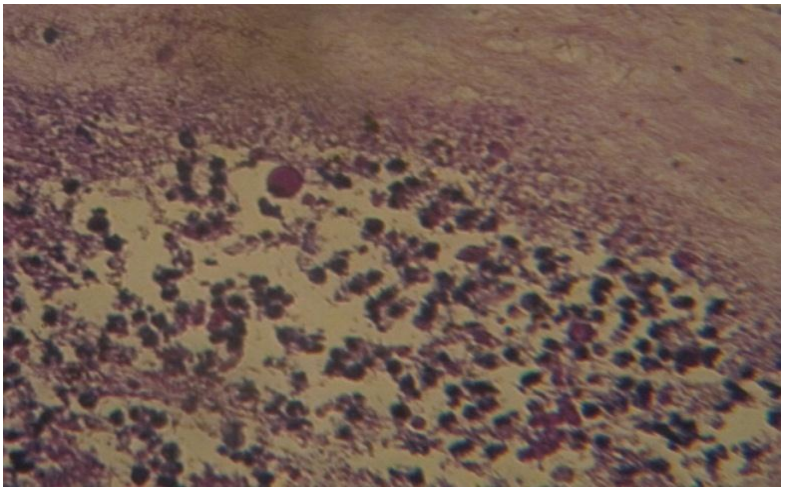


Fig:7. Histopathological section in the placenta of aborted sheep shows yeast in the necrotic debris (PAS stain 40X).

We demonstrated necrosis of chorionic villi with neutrophils infiltration, severe congestion of the blood vessels in the chorionic villous stroma, with neutrophils infiltration and sloughing and desquamation of trophoblasts .Also we recorded severe pathological lesions in the placenta in which Coccidioides immites isolated .

Discussion:

From the result, we recorded that, there is variation in the number of fungal isolates ,this may be due to bad managements to pregnant ewes that lead to immunosuppression of the pregnant ewe and lead to activate of the causative mycotic agents to induce abortion . Our result recorded that all collecting samples were positive for fungal isolates and the percentage of these isolates was different according to region of sample collection(Table:1),the higher percentage was recorded in the samples that were collected from al-manatheria,18 out 50,(36%) followed by AL-kuzweenia out 17(34%) and AL-shabaka ,15 out 50(30%). Our study was agreed with (13) who explained that fungal diagnosis depended on the morphological and microscopic examination.

These results may be due to the environmental condition provided proper condition to fungal infections as due to cold climate and poor nutrional condition, this result was consistent with Knadtson and Kirkbride,(14) who recorded that the incidence of mycotic abortion is sporadic, in the northern hemisphere, occurring mainly during the winter periods,when cows are usually fed with a large amount of hay .also Ali and Khan.(15) explained that the poor aeration and high humidity promote the environmental fungal growth. The administration of wet stored and mouldy fooder, in which a large amount of fungal spores could be present, is a further risk factor for the mycotic abortion. The fungal spores can penetrate through gastric lesions or the respiratory tract and reach the placenta and the fetus where there are optimal conditions for their full development (9).

We demonstrated that Aspergillus spp form 30% of fungal isolates, these results may be indicated that Aspergillus spp are the important causes of sheep abortion ,these result was in consistent with ,(16) who diagnosed mycotic abortion in five cases (3.4%) of bovine abortion in southern Brazil and he isolated *Aspergillus fumigatus* from four cases and *A. niger* from one, also (1)reported that fungal placentitis in bovine due to Aspergillus sp form 60-80% of mycotic abortion.

We demonstrated that Candida krusei and Candida zeylanoides form 6 out of 50 (12%) of fungal isolates ,these observation may indicated that other Candida .spp have a role in mycotic abortion in sheep particularly *C.krusei* that form(18%) of fungal isolation. Aziz,(17) reported that C .krusei constituted(4.08%) of fungi isolated from mastitis bovine milk .

The present study showed that Cryptococcus. uniguttulutus consisted the lower fungal isolates (4%) ,these results may be indicated that these species rarely associated with abortion, this idea was agreed with observation of ,(11) who explained that the Cryptococcosis is generally related to infection by *C. neformans* and *C. gattii* and is rarely caused by other species, *such as uniguttulatus*. (14) said that the other species of the genus *Cryptococcus* are generally considered to be saprophytes ,but later on, due to the increasing number of patients presenting compromised immune system and using of immunosuppressant agents ,the incidence of fungal infection by other *C.neoformans* and C.gatti has increased worldwide(18)

also (19)documented a case of equine cryptococcal endometritis and placentitis

in which the foal died on day 9 due to cryptococcal pneumonia.

The present study also reported that Rhodotella rubra was isolated from 5 cases of abortion,(10%),these observation may indicated that, these opportunistic fungi may be induced placentitis in sheep ,(20) explained that Rhodotorula species have emerged as opportunistic pathogens that have the ability to colonise and infect susceptible patients and induced fungemia that associated with meningeal infection ,in addition ,*Rhodotorula spp* infected lung and ear of sheep and cattle

According to our result ,we suspected that the fungi may cause abortion in the ewes and we suggested that in cases of ovine abortion ,the routine diagnostic procedures must be include the mycotic diagnosis.

We reported that *Coccidioides immitis* form high fungal (16%) isolates associated with abortion of the ewes. The morphological aspect of the colonies on Sabouraud agar, the microscopic aspect of the hyphae, sporangia and sporangiospores and the execution of the PCR confirmed the diagnosis of *Coccidioides immitis* in 8 cases of aborted ewe. However ,the isolation of *Coccidioides immitis* during the course of the study during spring season was supported idea that mentioned by Joseph,(21) who explained that the incidence of coccidioidomycosis is varable with season .Six out of 8 *coccidioides immites* isolates were identified in the placental samples that were collected from aborted ewes in Shabaka ,this area characterized by dry soil ,this evidence also was agree with Joseph ,(21) who found that the highest incidence of coccidiomycosis occur in dry soil.

The current study was considered a first study that reported *Coccidioides immites* in the Iraq .we suggested that *Coccidioides immits* reached the Iraq ,by travel infected patients from endemic regions in USA, these idea was agreed with Panackal *et al.,(*22) who mentioned that coccidioidomycosis may be reported in anywhere in the world due to , patients who acquired infection in the endemic region, can transmitted the disease to other region when they are travel to non-endemic areas

Molecular examination of TNF alpha gene

The results of gel electrophoresis of PCR products revealed the presence of -238bp band, in addition to the -300,400 and 600bp desired fragment(Fig:), from these results of PCR, we consider that, there was polymorphism in the TNF gene of aborted placental tissues ,and these results associated with fungal species isolates ,these results may be indicated that fungal infection of the placenta induced polymorphism of TNF-a genes ,these results were agreed with (23) who recommended that several immunogenetic polymorphisms in the human disease and HLA and cytokine gene are more relevant one ,also Roy et al.,(24) explained that variation in the TNF alpha promoter region has been associated with severe forms of malaria ,Leishminiasis ,meningococcal infection and Leprosy. ,these findings were supported by observations of Alvarez et al (25) who identified three polymorphisms in TNF-gene and also they recorded that the redesigned primers are shown below to amplify a TNF-alpha fragment that contains 90% of the original target sequence ,also other studies described two polymorphism in the human TNF alpha promoter at -308(19) and -238(26).

The present study showed that there is a relationship between the presence of multiple bands of TNF alpha gene by PCR with the presence of high intensity and score of TNF-alpha protein by immunohistochimistry(fig 3) ,.Some studies suggest that ,TNF gene polymorphism elicited production of TNF-alpha from immune and non-immune cells(27) ,this investigation was in

consistent with Wilson et al.,(28) who suggested that the polymorphisms in the TNF-303.2allele could act to increase constitutive and inducible levels of TNF-alpha.,.

We recorded that severe pathological lesion in the placenta that associated with high intensity of TNF-alpha ,which recorded by immunohistochemistry ,these results may be indicated that TNF-alpha play important role in inducing pathological changes in the placenta and may be considered an essential etiology of abortion in ewe, this investigation was agreed with Maria et al.,(29) who showed that the tumor necrosis factor-α (TNF-α) induced placental pathology, accompanying with fetal hypoxia, and neuroproliferative defects in the fetal brain

However, we recorded that a relationship between the result of TNF- gene by PCR and result of TNF-alpha protein by immunohistochemistry with the fungal infection that isolated from aborted placenta ,Also, this study reported high score of TNF alpha protein by immunohistochemistry in the aborted placenta in which *Aspergillus .spp .Coccidioidomyes spp,Candida* spp and *Cryptococcus* sp were isolated but low score of TNF-a were reported in the placental tissue in which other fungal spp were isolated ,these results may be indicated that, *Aspergillus.spp,Coccidioidomyces* sp.*Candida* spp and *Cryptococcus* spp, play an important role in induced placentitis and abortion in ewes while other fungal isolates might be not induced placentitis and abortion in the ewe.

The present study is the first report about the TNF gene polymorphism in placenta of aborted ewe in Iraq .

In the present study, macroscopic findings of the aborted placenta showed congestion necrosis, opaque, these findings were agreed with Johnson et al.,(30) who reported haemorrhagic necrotizing placentitis associated with thickened necrotic cotyledons in mycotic abortion of cattle,

microscopic examination of the placental samples revealed, necrosis of chorionic villi with neutrophils infiltration ,these results may be due to thrombus formation that occluded the blood vessels stream and lead to ischemia and necrosis . Necrotic tissue attract neutrophils into necrotic area ,this observation was coincidence with results of fungal isolation.

We also recognize yeasts in tissue section that stained by PAS stain ,this observation was in agreement with(13) who recorded the presence of mycotic elements associated with mycotic placentitis in cattle.

We found severe congestion of the blood vessels in the chorionic villous stroma ,with neutrophils infiltration and sloughing and desquamation of trophoblasts ,these results may indicated that infection is acute and the fungi invasion in the trophoblast may affect onn foetal growth and survive .this idea was agreed with (7) who explained that trophoblast play role in protective and nutrition of the foetus.

According to fungal isolation ,pathological lesions and detection of fungal elements in tissue sections ,we suggested that fungi play important role in abortion in sheep ,and on the base of more polymorphism bands of TNF-a gene, severe pathological lesion in the placenta in which Coccidioides immites isolated , we suggested that the Coccidioides immites is highly virulent and may induce abortion in the ewes.

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