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RESEARCH ARTICLE

Pathological, Immunohistochemical and Molecular Diagnosis of Abortions in Small Ruminants in Jordan with Reference to *Chlamydia abortus* and *Brucella melitensis*

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ABSTRACT

Abortion in small ruminants is a large problem in many countries in the world including Jordan and cause severe economic losses to sheep and goats producers. Definite diagnosis and identification of the abortive agent and its associated pathological lesions have not been fully described. Therefore, twenty-five formalin-fixed and paraffin-embedded sheep and goat placentas taken at the time of abortion were examined for the presence of *Chlamydia abortus* and *Brucella melitensis* using gross, histopathological examination, immunohistochemistry and quantitative polymerase reaction (qPCR) for each of these agents. Immunohistochemistry successfully identified the etiologic agents of *C. abortus* and *B. melitensis* in 13 and 3 placentas, respectively, with adequate visualization of organisms in expected locations and corresponding lesions. Quantitative PCR for both agents was positive in 14 of the 25 placentas, with 5 being positive for both agents at the same time, and infrequent correlation of IHC with qPCR results. It is proposed that qPCR may be an overly sensitive technique for abortion diagnosis due to chronicity of infection and use of live vaccines and needs to be associated with the pathological lesions.

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INTRODUCTION

Abortion in small ruminants induces high economic losses in many parts of the world, including Jordan (Samadi *et al.*, 2010). In the 2016 year, 2018 small ruminant abortion cases (1275 for sheep and 1143 for goats) were reported by the sheep and goat farmers to the Central Diagnostic Laboratories of the Ministry of Agriculture, and many other cases reported to the Veterinary Directorates in the different governorates in the periphery. Previously, serological and clinical data showed that *Brucella melitensis* and *Chlamydia* are the main causes of abortions in small ruminants (Al-Qudah *et al.*, 2004; Al-Ani *et al.*, 2004; Chu *et al.*, 2016). On the other hand, during a regional workshop which was conducted in 2014 at Jordan University of Science and Technology (JUST), regarding strategies to control abortions in sheep and goats, participants from several countries agreed that there has been no comprehensive study regarding the identification of abortions in these two species in the region (Brown *et al.*, 2014). However, it is very clear that there are multiple known causes of abortion, including both infectious and non-infectious (Holler, 2012).

For infectious agents, pathological examination and microbial identification can be frustrating. The placenta may not be available, or may have become severely autolyzed and/or contaminated. Similarly, fetal tissues are frequently too autolyzed for adequate pathologic interpretation. Very rarely the farmers submit both placenta and fetus together from the same aborted animal (Navarro *et al.*, 2009). Additionally, the placental lesions of inflammation and necrosis may be similar among abortion-causing agents so that attributing the histopathologic picture to one agent or another is not possible (Kalender *et al.*, 2013). Serology has been used for abortion diagnosis, but requires paired samples and this is problematic in having to find the same animal in an extensively raised herd or flock 2 weeks later for the second confirmatory sample. Furthermore, serology can be confounded by vaccination campaigns, which exist for some of the agents. Recently there has been a focus on molecular techniques but some authors caution that there may be sensitivity problems which engender false positives (Ilhan and Yener, 2008). Immunohistochemistry (IHC) is a technique that has proven useful for diagnostics and is applied in various laboratories for aiding diagnosis of abortions (Hazlett *et al*., 2013).

The aim of this study was to compare two diagnostic techniques – IHC and qPCR – for two of the known causes of small ruminant abortion in Jordan and the region, chlamydiosis and brucellosis, both of which are zoonotic (Saadi *et al*., 2010; Ababneh *et al*., 2014; Shahzad *et al*., 2017). It was hoped that one or both techniques could prove to be a reliable indicator for the identification of the causes of abortion in association with the placental lesions in order to more adequately understand the agents responsible for small ruminant abortion losses in the country.

MATERIALS AND METHODS

Sample collection: Samples for this study were all formalin-fixed (10%) and paraffin-embedded tissues from abortion material submitted to the Faculty of Veterinary Medicine at JUST. A total of 60 cases were available, but placenta was present in only 25 cases. The cases included placenta from 23 sheep and 2 goats. The same samples were used for hematoxylin and eosin (H & E), PCR and IHC examination.

Histopathological examination: Paraffin sections (4-5µm) were cut and stained by H & E method (Bancroft, 1990). The stained tissue sections were examined routinely by light microscope. The placental tissues were assessed for inflammation/necrosis (+ or -) and presence or absence of vasculitis (+ or -).

Immunohistochemical (IHC) examination: Unstained paraffin sections (4-5µm) were mounted on positively-charged slides; air dried, heated to 70ºC for 30’, then deparaffinized in xylene and allowed to dry thoroughly. Tissue was circled with PAP pen and endogenous peroxidase quenched with 3% H_2O_2, followed by running water wash. Antigen was retrieved by immersing the slides in Vector Unmasking Solution (Vector Laboratories, Burlingame) and heating in the microwave for 10min. Nonspecific binding was blocked with milk protein, followed by thorough washing in phosphate buffered saline with 0.02% Tween 20 (PBST, Bio Basic, Canada Inco). The primary antibody for *Brucella melitensis* was rabbit polyclonal, used at a 1:3000 dilution, and incubated at 37ºC for 2h. Secondary (biotinylated) goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was applied at a 1:250 dilution and incubated at 37°C for 1h. Streptavidin peroxidase (Elite-PO, Vector Laboratories, Burlingame, CA) was applied according to manufacturer’s instructions and incubated for 1h at 37°C. Substrate-chromogen was DAB (3,3’-diaminobenzidine) (Vector Laboratories, Burlingame, CA). When signal became evident, slides were washed in water for 10 min, counterstained with Mayer’s hematoxylin, dehydrated in xylene, and coverslipped with Paramount for a permanent record. For *Chlamydia* agent, a rabbit polyclonal primary antibody was used at a dilution of 1:2000 (ab21180) (Abcam, Cambridge, UK). Positive and negative control slides and antibodies were used.

Real time polymerase chain reaction: Total DNA was extracted from paraffin embedded placentas, using 8 sections, and was processed using commercially extraction kit and according to manufacturer’s instructions (QIAamp DNA FFPE Tissue Kit, QIAGEN Inc. Hilden, Germany). For qPCR, A QIAGEN fast probe master mix was used (QIAGEN, Hilden, Germany). This kit was used for the detection of the two pathogens. The primers and probe sequences for *Brucella* detection used were) (Gwida *et al*., 2011):

Forward primer: (5'GCTCGGTTGCCAATATCAATGC 3')
Reverse primer: (5'GGGTAAAGCGTCGCCAGAAG3')
Probe: (5'FAMACTCCAGAGCGCCCGACTTGATCG-TAMARA 3')

For the detection of *Brucella* spp, the qPCR reaction was composed as follows: 12.5µl of the QIAGEN fast probe mix, 2µl of each primer (10pmol), 0.25µl of the probe (10pmol), 2µl of the DNA sample and 8.25µl of nuclease free water to make the final volume to 25µl. The same reaction volumes were used for the detection of *Chlamydia* but with minor modifications. The primers and probes sequences used for *Chlamydia* were (Borel *et al*., 2008):

CpaOMP1-F: GCAACTGACACTAAGTCGGCTACA
CpaOMP1-R: ACAAGCATGTTCAATCGATAAGAGA
CpaOMP1-S: FAM-TAAATACCACGAATGGCAAGTT GGTTTAGCG-TAMRA

The qPCR conditions for each pathogen were as follow:

1. For *Brucella*: 1 cycle of 50°C for 2min, 1 cycle of 95°C for 10min, followed by 50 cycles of 95°C for 25s and 57°C for 1min (Gwida *et al*., 2011).
2. For *Chlamydia*: 1 cycle of 95°C for 10min, followed by 1cycle of 95°C for 15s and45 cycles of 60°C for 1min (Borel *et al*., 2008).

Positive results of qPCR were expressed as Ct value (Threshold value).

Statistical analysis: Data was analyzed using SPSS and χ^2 test. The analysis was limited to the data related to *Chlamydia* as the number of cases permits the analysis.

RESULTS

Histopathologic examination: Results are presented in Table 1. There was evidence of mixed inflammatory reactions of lymphocytes and neutrophils and necrosis in 92% of the examined placentas. In these cases, there was variable severity of degeneration/disruption of trophoblasts, and some had fibrin deposition within the outer (trophoblast) layers of the placenta. Eight of the placentas had mixed mononuclear inflammatory cell infiltrates visible within vessel walls (vasculitis) and in the vicinity of the blood vessels. In none of the cases were any organisms visualized by H & E staining.

Immunohistochemistry and qPCR: Table 1 displays results of the 25 placentas using immunohistochemistry and qPCR. Immunohistochemical signal for *Chlamydia* revealed trophoblasts filled with granular material positive for the organism in 52% of the placentas. The positive signal was found in the trophoblasts often as clusters of infected cells (Fig. 1 A-C). For *Brucella* immuno-histochemistry, positive signal was present in only 3 placentas, and here it was present in stroma and inside

Table 1: Detection of *Chlamydia abortus* and *Brucella melitensis* from 25 placenta tissues using histopathology, IHC and qPCR

	Species	Histopathology		IHC	qPCR
		Infl/necrosis	Vasculitis		
1	Sheep	+	-	CA	CA
2	Sheep	+	+	BA	CA, BA
3	Sheep	-	-	Neg	Neg
4	Sheep	-	-	Neg	CA, BA
5	Sheep	+	-	CA	CA
6	Sheep	+	-	CA	CA
7	Sheep	+	+	BA	CA
8	Sheep	+	-	CA	Neg
9	Sheep	+	-	CA	Neg
10	Sheep	+	-	CA	CA
11	Sheep	+	-	Neg	CA
12	Sheep	+	+	Neg	Neg
13	Sheep	+	-	Neg	Neg
14	Sheep	+	+	Neg	CA, BA
15	Sheep	+	-	CA	CA, BA
16	Sheep	+	-	CA	Neg
17	Sheep	+	-	CA	Neg
18	Sheep	+	-	Neg	CA, BA
19	Sheep	+	+	CA	CA
20	Sheep	+	-	Neg	Neg
21	Sheep	+	-	CA	Neg
22	Sheep	+	-	Neg	Neg
23	Sheep	+	+	BA	Neg
24	Goat	+	+	CA	CA
25	Goat	+	+	CA	CA

CA=*Chlamydia abortus;* BA=*Brucella melitensis*, Neg=negative and +=positive.

macrophages associated with the blood vessels. Signs were also seen in the inflammatory cells in some of the fetal lungs (Fig. 2A-D). There were no placentas that were positive by immunohistochemistry for both *Chlamydia* and *Brucella*. Nine placentas were negative by IHC for both the agents (36%). Overall, 28% of cases showed *Chlamydia* IHC positivity and were also positive with qPCR (Table 1). *Brucella* immunohistochemical signal was found in only 3 placentas, and of these, only one case was positive for *Brucella* by qPCR.

Twenty percent of the placentas were positive by qPCR for both *Chlamydia* and *Brucella*, and of these, one was positive for *Brucella* by IHC, one was IHC-positive for *Chlamydia* and 3 were negative by IHC for both agents (Table 1).

Analysis of the statistical results using χ^2 test, revealed that there is no statistically significant relationship (P>0.05) between IHC and real-time PCR results regarding *Chlamydia* cases. Analysis regarding *Brucella* was not performed as the number of cases was too small.

DISCUSSION

In this study, we successfully developed an IHC technique for *Brucella* and *Chlamydia* to identify the presence of the agents in formalin-fixed paraffin-embedded sections of placenta taken from small ruminants experiencing abortion. The immuno-histochemical signal visualized was distinct and consistent with what others have reported for appearance and extent of staining (Ahmed *et al.*, 2012; Rassouli *et al.*, 2013). In all immuno-histochemistry positive cases, there was evidence of damage and inflammation in the placenta, consistent with what the agent might cause. Additionally, to the 3 positive *Brucella*-IHC cases, there was evidence of vasculitis seen histologically, which is consistent with

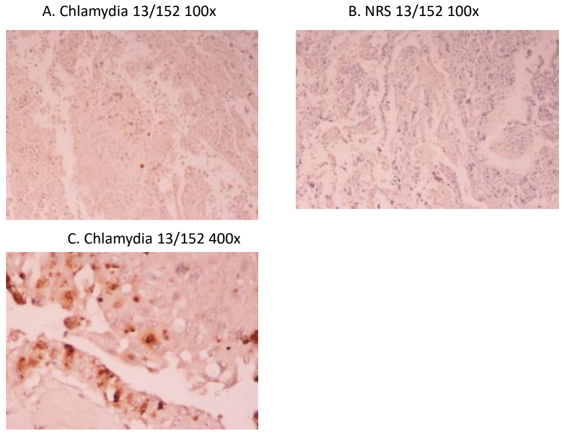

Fig. 1 (A-C): *Chlamydia*-specific immunohistochemical reaction: Immunoreactivity of the *anti- Chlamydia* polyclonal antibody to placenta tissues, areas labelled positive (brown) for C. abortus antigen in several trophoblasts. The reaction of *anti-chlamydia* antibody with the trophoplasts; A and C positive while B is the reaction with normal rabbit serum as negative control, (mag; A=100X, B=100X and C 400X).

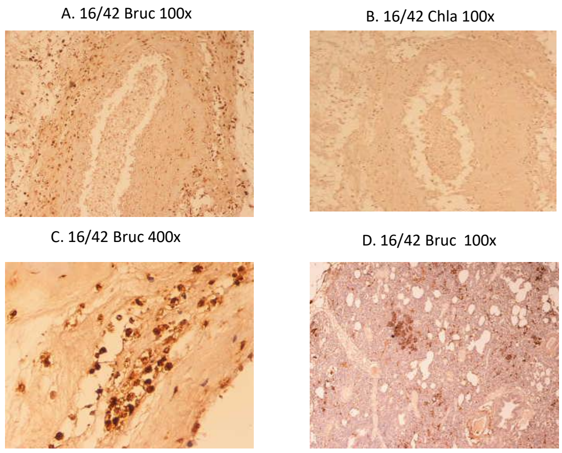

Fig. 2 (A-D): *Brucella*-specific immunohistochemical reaction: Immunoreactivity of the *anti-brucella* polyclonal antibody to placenta and lung tissues, areas labelled positive (brown) for Brucella antigen in several macrophages infiltrating the wall of the blood vessels (vasculitis) A, in the stroma of the inter-cotyledon C and the fetal lungs D. The reaction in plate B is reaction with normal rabbit serum as negative control, (mag; A=100X, B=100X, C400X and D=100X).

pathogenesis of this agent (Plumeriastuti and Saad, 2012; Nagati and Safaa, 2016). Based on the presence of the agent in sufficient quantity to detect by IHC and the corresponding lesions for corroboration, there is good indication that the IHC technique has accuracy for determining the cause of abortion, as others have reported (Navarro *et al.*, 2009; Neta *et al.*, 2010; Gul *et al.*, 2013).

The results of the qPCR using formalin-fixed and paraffin-embedded sections are far more difficult to interpret. The qPCR results were often conflicting with the IHC results; both agents positive in the same placenta, positive qPCR result when neither immunohistochemistry was positive, negative qPCR results when IHC tests were positive and positive qPCR results for one agent when the IHC test was positive for the other agent.

Several of these problems can be explained by the nature of these two infections. Both *Brucella* and *Chlamydia* are persistent infections. They usually cause abortion only once, and although the animal remains infected perhaps for life, for the next pregnancy, usually there is a sufficient immune response that the agent cannot gain an adequate foothold in the placenta to generate enough damage to lead to abortion (Diaz, 2013). Because the agents are continually present, an extremely sensitive test such as real-time PCR could remain positive on almost any tissue in the body of animals for a long period of time of their life (Gwida *et al.*, 2016). An additional problem of positive qPCR results in the presence of negative IHC might be explained through vaccination. Most farmers in Jordan vaccinate against brucellosis using live attenuated Rev-1 vaccine, and in previous studies, the vaccine strain was found in the aborted feti (Smirnova *et al.*, 2013; Dougherty *et al.*, 2013; Wareth *et al.*, 2014, 2015).

Other investigators have found similar issues with qPCR as a means of diagnosing abortion. Hazlett *et al.* (2013) performed IHC and qPCR for both *Chlamydia abortus* and *Coxiella burnetii* on cases of sheep and goat abortions and found that both agents were frequently identified when the actual cause of abortion, as defined by the pathologist and ancillary testing (bacterial culture, ELISA, IHC for Toxoplasma) was different from the qPCR result. They concluded that qPCR may be too sensitive a technique for determining the cause of abortion, and qPCR results should be associated with pathological lesions. It is also possible that the false positive cases with qPCR are due to contamination of tissue samples during collection in the farm.

The negative cases with PCR and IHC results are also deserving of attention. For *Chlamydia*, it is known to cause damage in the placenta in a segmental fashion, i.e., there will be a focal section of the cotyledon seriously affected or damaged by extensive proliferation of the organism. It is possible that the sections taken for the qPCR simply did not contain the agent of concern.

Conclusions: Immunohistochemistry holds excellent promise for usefulness as a technique to pinpoint the etiology in cases of *Chlamydia* and *Brucella*-induced abortions. Caution should be exercised in interpreting qPCR results from cases of abortion, as the technique may be too sensitive to allow for attribution of the agent as the etiology of the abortion. We recognize that there are many other potential causes of infectious abortion in sheep and goats, including *Toxoplasma*, *Campylobacter*, *Coxiella*, and others such as akabane, border and Schmalenberg viruses, but we did not include those agents in our study. They may well have been responsible for the cases that were negative by both IHC and qPCR in our study, or perhaps the abortion was not the result of an infectious agent.

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Authors contribution: NH formalized the research idea, writing the proposal and the analyzing the results and writing the manuscript. SK collected the samples, preparing slides, run IHC and PCR. MA supervised the PCR examinations. CB discussed the concept, reading some slides and developing IHC and reviewing the manuscript.

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