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Study the effects of *Corynebacterium pseudotuberculosis* antigens in the improvement of fertility in female mice against the virulent pathogen with virulent type of same pathogen

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Abstract

This study was designed to detect the efficiency of immunization with culture filtrated antigen (CFAg) and whole sonicated antigen (WSAg) prepared from a local strain of *Corynebacterium pseudotuberculosis* and improvement of the fertility in female. Eighty female mice were divided into 4 groups equally: Group1 & G2 of mice were immunized with (CFAg) & (WSAg) respectively at dose 0.3 ml subcutaneously in Two doses, 2 weeks interval of each Ag. Group 3 served as a control. At 28 days post immunization skin test was done, then at 30 days blood sample used for the serum interferon Y Elisa test. The mice in G1, G2, and G3 were challenged virulent *C. pseudotuberculosis* (5×10^9) CFU/ ml I/P. At 30 days post infection, 10 mice from each group were scarified to determine serum hormones (estrogen, progesterone, FSH and LH), record the numbers of ovarian follicles, in addition to this; small pieces from ovary, oviduct and uterus were processed for histopathology. The results revealed that the antigens stimulate cellular immunity in immunized mice. The serum levels of estrogen, progesterone were high and FSH and LH were low levels in G3 as compared with those good values of G1 & G2 in addition, there is variation in the number of types of ovarian follicles between the mice groups. This study concluded that immunization with *C. pseudotuberculosis* antigens leads to increase immunity that lead to protect of genital female organs in immunized mice in comparison with infected mice which caused different changes which may lead to infertility.

Keywords: *C. pseudotuberculosis*; CFAg; WSAg; infertility; reproductive hormones

Introduction

Corynebacterium pseudotuberculosis is the causative agent of Caseous lymphadenitis (CLA) in small ruminants ^[1,2]. CLA mainly characterized by suppurative abscesses in both superficial and visceral lymph nodes ^[3]. *Corynebacterium pseudotuberculosis* infected cattle, camels, buffaloes, horses, pigs and deer as well as laboratory animals ^[4]. The defense mechanism of *C. pseudotuberculosis* prevents the organism from being isolated in all bacteriology cultures ^[5].

Corynebacterium pseudotuberculosis may be called a "perfect parasite" because of its ability to evade immune system with apparent ease when established within the host. *C. pseudotuberculosis* has a gene responsible for long-term survival outside the host environment ^[6]. Immune responses against *C. pseudotuberculosis* are complex and including both arms of immunity, cellular and humoral immune response ^[7,8]. Also Paule *et al.* ^[9] was recorded that high and medium or low patterns of response of INF Y in sheep and goat infected with *C. pseudotuberculosis*, at five days and secondary response occurs on day 16 then decrease in 42 to 56 days post infection. Also ^[10] who considered the production of INF Y as a highly specific indicator of CLA. While ^[11], who concluded that the attenuated T1 strain of *C. pseudotuberculosis* induce both humoral and cellular immune responses in BALB/c mice, also they found that the 10^7 CFU dosage did not show any lesions in mice, this demonstrated that there was production of antibodies, but the cellular immune response is important to the control of CLA.

In spite to the low mortality rate induced by *C. pseudotuberculosis* but it causes important economic losses associated with infertility problem in animals ^[12]. Which include decrease number of birth offspring ^[13], imbalance production sex hormones ^[14], reproductive failure ^[15], and impairment of reproductive hormones in mice ^[16,17,18].

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Chronic infection of goats by *C. pseudotuberculosis* lead to impaired production of estrogen and progesterone, the maintaining of pregnancy was dependent on the activity of the progesterone, in addition, these hormones and estrogen can influence on the estrus cyclic function which expressed relationship with hypophysis gonadal [19,20]. Infectious disease cause oxidative stress which release by neutrophils and macrophages in respiratory burst reaction in order to destroy the pathogen in the phagosome [21, 22], also ROS generation associated with releasing Calcium ions from ER that lead to damage of mitochondria and stimulated inflammatory reaction [23].

In Iraq, there are a few studies about the influence of *C. pseudotuberculosis* on female fertility, therefore the aims of the present study used to determine the efficiency of two antigens CFAg and WSAg in improvement females mice fertility post infection with *C. pseudotuberculosis*.

Materials and Methods

Bacterial Strain: The strain of *C. pseudotuberculosis* isolated from a case of CLA in sheep. This strain was diagnosed previously by bacteriological method and PCR assay by Al Badrawi [24] in the laboratory of Internal and Preventive Veterinary Medicine Dept./College of Veterinary Medicine/ University of Baghdad.

Preparation of *Corynebacterium pseudotuberculosis* infective dose (challenge):

Challenge dose was prepared from the bacterial suspension of *C. pseudotuberculosis* according to [12], calculation and adjustment of this dose to 5×10^9 CFU/ml according to Miles and Misra [25].

Whole sonicated antigen (WSAg) and soluble antigen:

This antigen was prepared according to Mitove *et al.* [26]. The microorganism was cultured on (TSA) and incubated at 37 °C for 72 hours, then the growth was harvested by adding 5ml of PBS, Centrifuged of suspension at 3000rpm for 30 minutes at 4°C, then the sediment washed three times with phosphate buffer saline (PBS) (pH=7.2) and resuspended with PBS. Prepared bacteria were sonicated at 60 Hz, using five cycles of 60 s each (Branson Sonifier 450). Post sonication, bacteria was centrifuged for 30 min at 10,000 g/ 4 °C, the sediment was used for immunization and the supernatant was frozen at 20 °C until used in skin test. Protein concentration in the two antigens was measured using biuret kit according to Henry *et al.* [27].

Culture filtrate Ag (CFAg): This antigen was used for immunization, prepared by modified method according to Vale *et al.* [11].

Experimental Design

Forty five female and twenty male mice were used in this study. The eighty (BALB/ C Strain) female mice, aged 8-10 weeks and weighed 20-25gm were divided into 4 groups equally in addition to 20 male mice used at the same age, each group, treated as the following.

***G1** (n=15): Immunized with culture filtrated antigens of *C. pseudotuberculosis* (CFAg), S/C, two doses at two week intervals. (Protein concentration 2mg/ ml).

***G2** (n=15):Immunized with whole sonicated antigen (SC Ag) S/C, two doses at two week intervals. (Protein concentration 2mg/ ml).

***G3** (n=15). Administrated orally with 0.3 ml of normal

saline and served as a control group.

At 28 days post immunization skin test was done to the mice in G1, G2 & G3 and At 30 days 5 mice from G1, G2 and G3 were scarified and blood collected for measurement of interferon Y in serum. Then the remainder mice in G1, G2 & G3 were challenged with (5×10^9 CFU/ml) of virulent *C. pseudotuberculosis*.

At 30 day post infection 10 animals from each group were scarified to collect blood for estimation of hormonal level (estrogen, progesterone, F.S.H and L.H), counting of ovarian follicles, in addition to taken small pieces of ovary, oviduct and uterus for histopathological examination according to Luna [28].

Determination numbers of stillbirth and offspring fetus:

The numbers of stillbirth and offspring fetuses in all groups were calculated according to the following equation [29].

Fertility index= No. of pregnant female/ Total no. of conception mating x100%.

Pregnancy index= No. of female gives full term birth/ No of pregnant female x 100%.

Statistical Analysis

All data were represented as means + SE. One way analysis of variance (One-way ANOVA) by using SPSS program, The level of statistical significance was set at ($P < 0.05$) [30]

Ethical approval: This study was approved by the ethical and research committee- College of Veterinary Medicine- University of Baghdad, Ministry of High Education and Scientific Research.

Results and Discussion

Skin Test

The results showed that the main difference of skin test was significantly higher ($P \leq 0.05$) in the immunized groups (G1 & G2) at 48 hours, which revealed (1.7 ± 0.20 and 1.5 ± 0.30) respectively, while control negative group (G4) didn't show any reaction (Table 1).

Table 1: Skin thickness differences of immunized animals against soluble *C. pseudotuberculosis*

Groups	At 24 hours	At 48 hours	At 72 hours
G1	0.8 ± 0.06 B	1.7 ± 0.20 A	0.9 ± 0.10 B
G2	0.7 ± 0.70 B	1.5 ± 0.30 A	0.8 ± 0.04 B
G3	0	0	0

Different capital letter means significant at ($P \leq 0.05$).

Levels of IFN γ .

The current results showed that the values of serum IFN γ in G1 and G2 were higher (422 ± 1.0 & 241 ± 1.50) Pg/ml, respectively than the value in control negative group (51.50 ± 0.60) Pg/ml at 30 days post infection. A significant result appeared in G1 than in G2 and G3 (Table:2).

Table 2: Mean values of serum IFN γ level in immunized and control -ve mice at post 30 days.

Group	IFN Y (Pg/ml) (Mean \pm SE)
G1	422 ± 1.0 A
G2	241 ± 1.50 B
G3	51.50 ± 0.60 C

Different capital letter means significant at ($P \leq 0.05$).

Female reproductive hormones at day 30 post challenge:

The results in table (3) revealed the means of serum estrogen,

progesterone, FSH and LH hormones in mice at 30 days post challenge, in G3 there is a significant increase in Estrogen and

progesteron hormones in the control group (Table 3).

Table 1: Values of serum levels of hormones in immunized animals at 30 days post infection.

Groups	Estrogen (Mean ± SE)	Progesterone (Mean ± SE)	FSH (Mean ± SE)	LH (Mean ± SE)
G1	40.1±0.45 C	34.8±0.66 C	13.51±0.089 A	4.26±0.050C
G2	44.3±0.70 B	44.2±0.50 B	10.30±0.041 B	4.07±0.025 C
G3	73.8±0.90 A	59.3±0.89 A	8.5±0.320 C	4.70±0.270 B

Different capital letter means significant ($P \leq 0.05$).

Ovarian follicles and Corpus Luteum at 30 days post infection

The results expressed that the number of primordial follicles in the mice of G1 was (21) more than G2 mice (16), also more

than mice of G3 and G4. Also the results showed that the numbers of atresia follicles in G3 were high (21) more than the two immunized groups and control negative group (Table: 5).

Table 5: Number and types of ovarian follicles in all groups at 30 days post infection

Mice Groups	Number and type of ovarian follicles in all groups						
	primordial	primary	secondary	Tertry	graafian	atresia	Total number
G1	21A	7C	4C	5C	1E	4C	42
G2	16B	6C	5CD	6C	3D	12B	48
G3	13B	7C	5D	3D	0F	21A	49

Different capital letter means significant ($P \leq 0.05$).

Histopathological examination post 30 days of infection In Non immunized infected animals

Ovary: At 30 days post infected with *C. pseudotuberculosis*, the ovary expressed a large number of atresia ovarian follicle characterized by neutrophils infiltration and vacuolation granulosa cells with cellular debris in antrum in addition to primordial follicle (Fig: 1).

Oviduct: The oviduct expressed vacuolation & hyperplasia of epithelial cells with inflammatory cells particularly neutrophils infiltration in its wall and lumen with erosion of epithelial cells (Fig: 2).

Uterus: The uterus showed mucus in its lumen with focal desquamation of epithelial layer in addition to hyperplasia of epithelial cells, inflammatory cells particularly neutrophils in its lumen (Fig: 3).

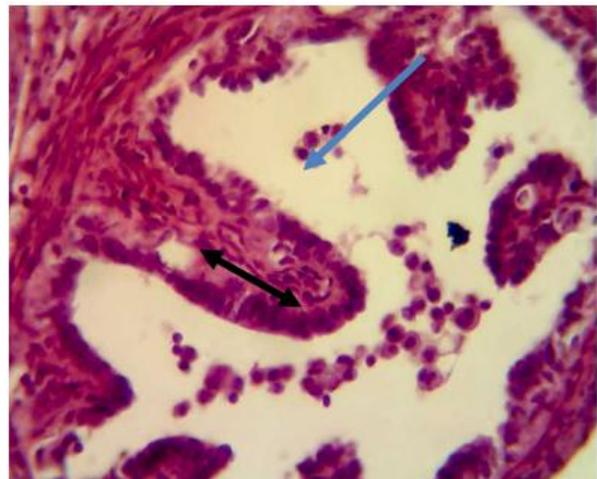


Fig 2: Section in the oviduct of mouse at thirty days post infected with *C.pseudotuberculosis* shows inflammatory cells particularly neutrophils in its lumen (←) with erosion (↔) and vacuolation of epithelial cells (←) (H&E stain 400X).

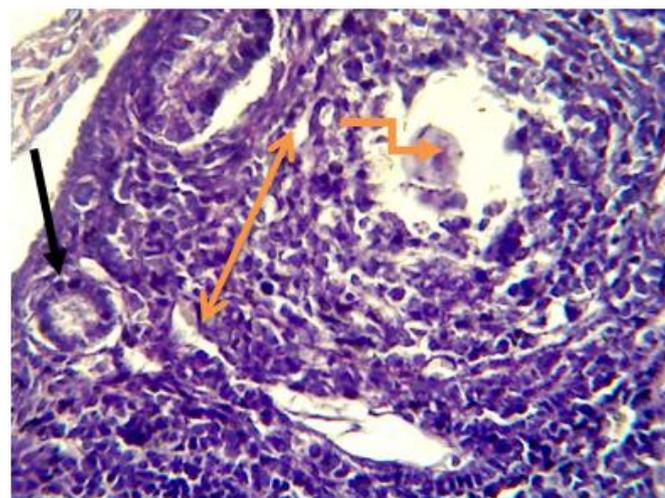


Fig 1: Section in the ovary of mouse at thirty days post infected with *C.pseudotuberculosis* shows primordial (→), primary, secondary follicles (↔) in addition to atresia ovarian follicles characterized by vacuolation and necrosis of granulosa cells with cellular debris in antrum (↔) (H&E stain 400X).

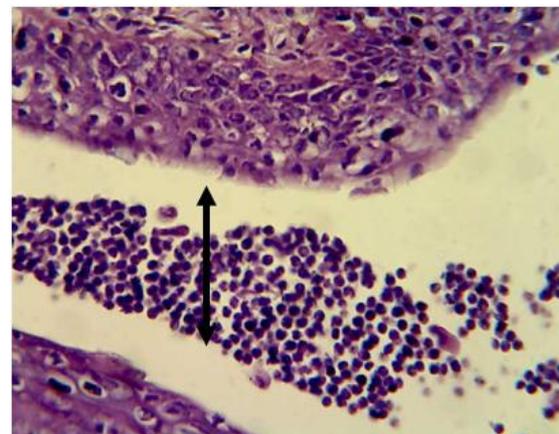


Fig 3: section in the uterus of mice at thirty days post infected with *C.pseudotuberculosis* shows hyperplasia of epithelial cells with neutrophils in the lumen (↔) (H&E stain 400X).

Pathological changes in Immunized animal with CFAG post 30 days of challenge

Ovary: No lesions appeared in ovary (Fig: 4).

Oviduct: The oviduct of immunized mice by CFAG at thirty days post infected with *C. pseudotuberculosis* shows no lesions (Fig: 5).

Uterus: The uterus revealed no lesions and in other animals, it was reported high cellularity of the subepithelial layer (Fig:6).

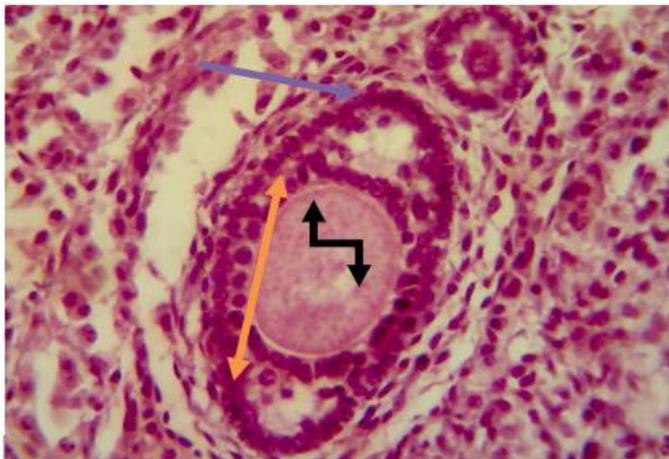


Fig 4: Section in the ovary of immunized mouse by CFA ginfected with *C. pseudotuberculosis* shows primary follicle consist from single cubodial layer of granulosa cells around antrum containing oocyte (———), in addition to tertiary follicle expressed two cavity of antrum (———) surrounded with granulosa cells, theca interna (———) and no lesions (H&E stain 400X).

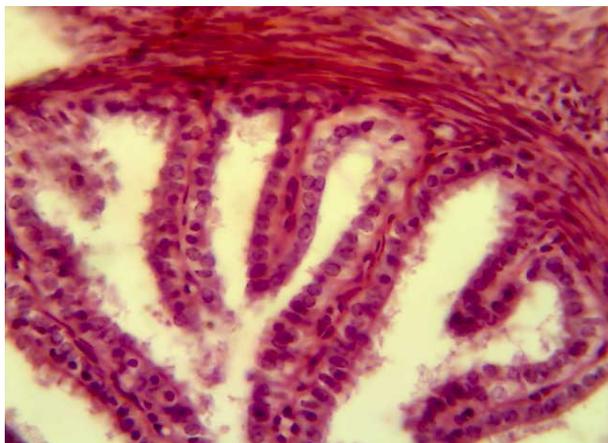


Fig 5: Section in the oviduct of immunized mouse by CFAG at thirty days post infected with *C.pseudotuberculosis* shows no lesions (H&E stain 400X).

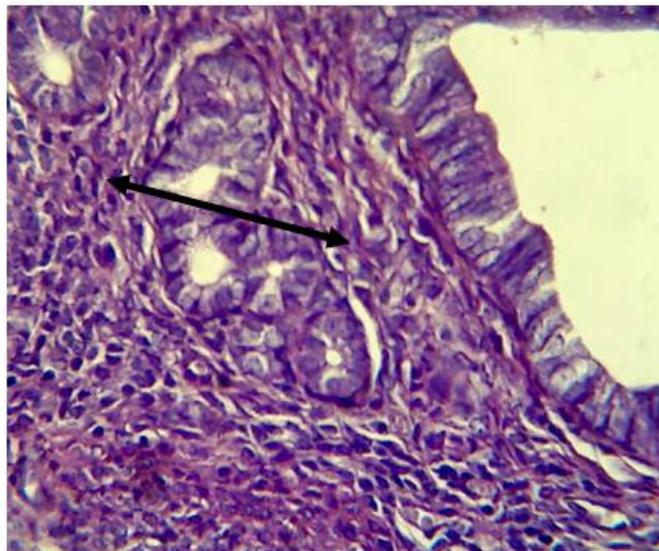


Fig 6: section in the uterus of immunized mouse by CFAG at thirty days post infected with *C. pseudotuberculosis* shows high cellularity of subepithelial layer (———) (H&E stain 400X).

Pathological changes in Immunized animals with whole sonicated antigen post 30 days of challenge

The oviduct section:- Immunized mice by WSAg at thirty days post infected with *C. pseudotuberculosis* showed few mucus in the lumen of the oviduct (Fig: 7).

The uterus:- The lesions expressed few inflammatory cells infiltration and edema in the endometrium (Fig: 8), in other animals, there were no lesions in the uterus.

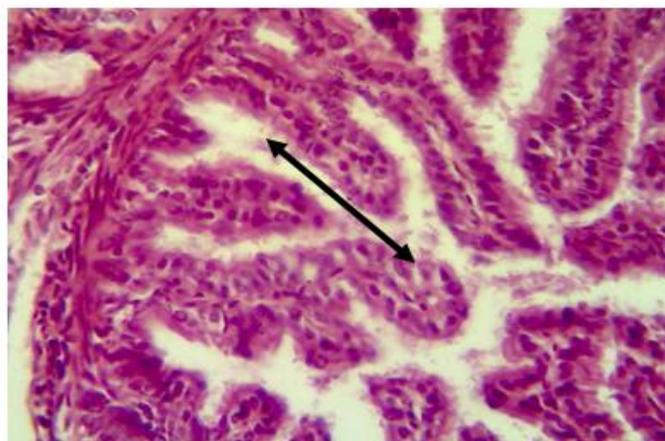


Fig 7: Section in the oviduct of immunized mouse by WSAg at thirty days post infected with *C. pseudotuberculosis* shows few mucus in its lumen (———) (H&E stain 400X).

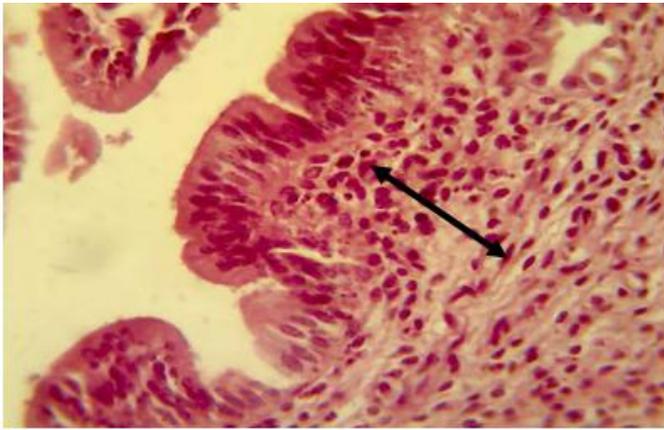


Fig 8: Section in the uterus of immunized mouse by WSAg at thirty days post infected with *C. pseudotuberculosis* shows few inflammatory cells infiltration and edema in the endometrium (↔) (H&E stain 400X).

Discussion

Skin Delay type Hypersensitivity reaction (DTH) in the present finding may indicate that CFAG and whole sonicated antigens of *C. pseudotuberculosis* can stimulate cell mediated immune response because DTH was an important arm of the immune response, the present evidence was an agreement with observations of Ghani & Habasha [31], who reported that cell mediated immune response play a major role in protection and providing adequate protection against CLA. Also Salema *et al.* [32] was concluded that immunized mice with Brucella CFAG induce DTH skin reaction.

The current finding revealed high levels of serum INF Y in immunized animals, these results may indicate that CFAG can stimulate CD4 and CD8 T cells secreted INF Y that was responsible for DTH reaction, these ideas was consistent with Trinchieri [33] concluded that a clear picture is emerging that IL-10 is involved in limiting inflammation although often also in preventing sterile cure in chronic infection by intracellular parasites. And a study of Fortune *et al.* [34] concluded that stimulated macrophages and dendritic cells can produce IL12 which stimulated Natural killer cells to produce INF γ .

The present finding revealed that non immunized infected females (G3) expressed high levels of serum estrogen and progesterone as compared with those values in other groups post infection, these results may be due to severe infection of the uterus tissue and ovary that stimulated these tissue to produce estrogen and progesterone, these idea was in consistent with Othman *et al.*, (2014) [19], who reported changes in sex hormonal levels in goats infected experimentally with *C. pseudotuberculosis*. Abnormal arise in these hormone may associated with a defect in normal development of ovarian follicular; this idea was in agreement with an investigation of Othman *et al.*, 2016 [35], who mentioned that CLA infection in non-pregnant does may lead to infertility resulting from pathological changes in the reproductive organs as well as an imbalance in reproductive hormonal levels.

The current result expressed low levels of estradiol and progesterone hormones in immunized infected animals particularly in those animals immunized with CFAG as compared with non-immunized and immunized animals with WSAg post infection, these result may indicated that CFAG provide a good immunity against infection that prevent or decrease load of infection that decrease the levels of production of these hormones since infection by these pathogen lead to increase levels of serum progesterone and

estradiol in the current finding which revealed that the levels of serum FSH in immunized infected animals were high than those in non-immunized infected animals, on the base on above observation, it was suggested that CFAG and WSAG may prevent bacterial infection associated with decrease in the generation of oxidative stress that destroyed pituitary glands, these evidence was in consistent with Hall [36] who demonstrated that the oxidative stress associated with *C. pseudotuberculosis* infection can cause damage of the main source of FSH and LH hormones, anterior pituitary gland, which mediated by hypothalamus producing gonadotropin releasing hormone (GnRH). On basis of the above results, it was suggested that one important marker of healthy condition of females reproductive system were levels of serum FSH, estradiol and progesterone [37].

The present finding demonstrated that the number of ovarian follicles were high in non-immunized infected animals associated with high number of atresia follicles as compared with other groups, these result was agreed with high levels of serum estradiol and progesterone in these group, these result indicated that the FSH and LH hormone are essential in maturation ovarian follicles, this idea was in agreement with observation of Woodruff *et al.* [38], who reported that LH act synergistically with FSH hormone in folliculogenesis.

The absence of pathological lesions in the reproductive tract of immunized mice post infection as compared with those females in positive group may indicate that immune response may prevent bacterial dissemination, these ideas were consistent with Hodgson *et al.* [39], who suggested that anti PLD antibodies can abolish toxin induced vascular permeability which prevent bacterial dissemination. The culture filtrate Ag is consistent from bacterial secretion such as PLD and other protein that mediated immune response against bacterial virulence factors, this evidence was agreement with Walker *et al.* [40] and Droppa-Almeida *et al.* [41], they demonstrated that 40 kDa serine protease, a protein secreted by *C. pseudotuberculosis* can stimulate protective immunity against this pathogen in sheep.

Conflict of interest

The authors declare no conflict of interest

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Conclusion

This study concludes that the infection with virulent type of *C. pseudotuberculosis* caused female infertility with low gestation index, Fertility index and died of all offspring while the immunization of mice by two antigens, including CFAG and WSAG prepared from local strain of *C. pseudotuberculosis* can increase the immunity and improvement of the animals' fertility with high percent of gestation and live of the offspring at 100%.

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