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IMPROVEMENT OF MICE FERTILITY BY IMMUNIZATION WITH CULTURE FILTRATED *CORNYEBACTERIUM PSEUDOTUBERCULOSIS* ANTIGEN

[1]Awni, K.J., [1]Alwan, M.J., [2]Afaf Abdulrahman Yousif
[1]Department of Pathology and Poultry Disease, College of Vet. Medicine, University of Baghdad, Iraq;
[2]Department of Internal and Preventive Veterinary Medicine, College of Vet. Medicine, University of Baghdad, Iraq.
Corresponding author email: - afaf_a.rahman@yahoo.com

ABSTRACT
This study was designed to improve the fertility in immunized mice, which were infected with virulent *Corynebacterium pseudotuberculosis* forty male mice were divided into 3 groups Group 1:15 mice were immunized with *C. psuedotuberclosis* culture filtrated antigen (CFAg), 0.3 ml subcutaneously in Two doses, 2 weeks interval. Group 2:10 mice were served as positive control. Group 3:15 mice were served as negative control. Twenty eight days post immunization skin test was done and post 30 days 5 mice group 1 and 3 were sacrificed for blood collection to measurement IgG and INF ɣ in serum by ELISA test. Thirty days post immunization group 1 and 2 were infected (challenged) intraperitonially with 0.3 ml of virulent *C. psuedotuberclosis* containing (5×10^9) CFU/ ml. Thirty days post infection, 5 mice of each group were scarified to measure of IgG, INF ɣ and level testosterone inserum and examine morphology of sperm as well as pathological lesion of male reproductive tract, the other 5 mice were mixed with normal females (one male with two females) for 10 days to determine the gestation index %and numbers of offspring from each group post 60 days. The results showed increased skin thickness in skin test and an increase in INF ɣ with stimulation of humoral immune response by an increase in IgG in the immunized group only post immunization and infection. A decrease in testosterone hormone and in number of sperm in the infected animals post 30days of infection by virulent bacteria while the immunized animals showed higher testosterone and live sperm count. Also showed improved in fertility 100% in comparison with the infected non immunized animals post mating of mice and the pathological changes showed necrosis of all epithelial cells of seminiferous tubules as well as incomplete spermatogenesis, low number or no sperms in the infected mice. No changes appeared in the testis of immunized mice with complete spermatogensis and epididymis lumen was filled with sperms. The percentages of pregnant index in normal female mating with infected male with *C. pseudotuberculosis* was (0%) as compared with those values in femal mated with immunized males with CFAg (100%) post 60 days from infection. This study concludes that the animals infected with *C. pseudotuberculosis* lead to infertility while immunization with CFAg of *C. pseudotuberculosis*lead to improvement of fertility 100%.

KEY WORDS: -*C. pseudotuberculosis; CFAg*; infertility; Mice.

INTRODUCTION

Caseous lymphadenitis (CLA) is an important chronic disease of small ruminants, large animals and human cause by *Cornybacterium pseudotuberculosis* (Costa *et al*., 2017). The main features of this pathogen are gram positive small curved bacillus intracellular facultative anaerobic microorganism (Jesse *et al.*, 2011). This organism can be transmitted to the hosts by different routes, including oral, intranasal, intraperitoneal and intradermal inoculation (Othman *et al*., 2014).

Caseous lymphadenitis was taken two forms the first form an external form which characterized by presence of abscesses in the superficial lymph nodes and subcutaneous tissue and the second form was internal form in which the internal organs were suffering from abscesses particularly lung, liver and kidney in addition to mediastinal and bronchial lymph nodes (Azda-Rina *et al*., 2013). The virulence factors of *C. pseudotuberculosis* are dependent on the ability to produce phospholipase D hydrolyzing spingomyelin of cell membrane, particularly endothelial cells that facilitated spread the infection in host tissues, in addition to the ability of acquision of iron through fag operon gene that help intracellular bacterial survival (Foster, 2013). These pathogens also able to avoid host immune response and causing necrotizing granulomatous lymphadenitis (Junior, 2006). *C. pseudotuberculosis* modifies its proteomic profile in the laboratory versus infection conditions and adapts to the host context during the infection process. The screening proteomic performed us enable identify known virulence factors, as well as potential proteins that could be related to the virulence of this pathogen (Silva *et al.*, 2017). *C. psuedotuberclosis* cause asymptomatic infection that leads to failure diagnosis of infected herds associated with widely spread the infection from animal to animals as well as transmission of the infection to the human (Brown and Olander, 1987). Improper removed of the abscesses, using disinfectants as well as antimicrobial treatment help in the spreading the disease in the environment as a result of rupture of the abscess (Washburn *et al*., 2009; Santiago *et al*., 2010). Particularly this pathogen was resistant to most antimicrobial agents due to their ability to biofilms formation in addition to thickness fibrous connective tissue capsule around the abscesses (Braundmeier *et al*., 2015). This pathogen cause animal infertility due to infection by this pathogen lead to increased levels of estrogen and progesterone, which lead to impairing ovulation and implantation, which finally led to cull the

infected animals from breeding flocks (Williamson, 2001; Othman *et al*., 2014). *C. pseudotuberculosis* can reach the reproductive tract through the afferent lymphatic system before proliferation in the macrophages and this pathogen can survive the environment of phagolysosomal enzymes (Dorella *et al*., 2006; Baird and Fontaine, 2007). Caseous lymphadenitis disease was very difficult to treated and limiting efficiency of herd management programs in the protective the animals from opportunities infection of external wounds, therefore the important programs in the controlling this disease is vaccination of the herds (Abdullah *et al.,* 2013; Moussa *et al*., 2016). There are no available data about the efficient vaccine prepared from local strains of *C. pseudotuberculosis* against infection by this pathogen, thus the aim of the this study was to identify the efficacy of culture filtrate antigens of the *C. pseudotuberculosis* in for improvement of fertility in male mice challenge by a virulent strain of this pathogen.

MATERIALS & METHODS

Bacterial strain

The strain of *C. pseudotuberculosis* was obtained from Dept. of Internal and Preventive Veterinary Medicine/ College of Veterinary Medicine. The bacterium was isolated from acase of CLA in sheep and diagnosed by conventional microbiological methods, including gram stain, synergistic hemolytic CAMP reaction with *Rhodococcus equi*, morphology of the colonies, biochemical test (catalase and urease production), in addition the ability to ferment glucose and maltose and finally confirmed by PCR assay by (Al Badrrawi, 2016).

Preparation of *Corynebacterium pseudotuberculosis* infective dose:

To prepare the bacterial suspension of *C. pseudotuberculosis,* the bacteria inoculated into 10 ml of brain heart infusion broth and incubated at 37 °C for 72 hours, then centrifuged by cold centrifuge at 3000 rpm for 30 minutes, the sediment washed three times with phosphate buffer saline (PBS) (pH=7.2) and re-suspended in 5ml of PBS, adjusting the bacteria to 5×10^9 CFU according to (khuder *et al*., 2012). The infective dose of bacteria was calculated according to (Miles and Misra, 1938).

Preparation of soluble antigen: The soluble antigen was prepared According to the (Motive, 1992). The protein concentration was measured using biuret kit according to (Henry *et al*., 1974).

Culture filtrate Ag (CF Ag): This Antigen was prepared by modified method of (Vale *et al.,* 2016).

EXPERIMENTAL DESIGN

Forty Male mice average age (8-10 weeks) were divided into 3 groups and treated as follows:

G1(Immunized group):15 male mice were immunized with culture filtrated antigen (CF Ag) of *C. psuedotuberclosis* at dose 0.3ml in Two doses, 2 weeks interval.

G2: 10 male mice were served as positive control.

G3: 15 male mice were served as negative control.

Twenty eight post immunization skin test was done and 5 males from first and third group were scarified and blood was collected for measurement serum level of interferon gamma (INF Y), immunoglobulin G (IgG) and testosterone. Then the mice from G1 and G2infected (challenged) intraperitonially with 0.3 ml/of bacterial suspension of virulent *C. psuedotuberclosis* containing (5×10^9) CFU/ ml.

Thirty days post challenge, 5 animals from each group were scarified and blood was collected for measurement of serum (INF ɣ), (IgG), testosterone, Sperm count and histopathological changes of testis and epididymis (Luna, 1968).

The remaining animals of G1, G2 and G3 were mixed with normal females at ratio 1:2 for 10 days and then spread from each group to determine the gestation index with numbers of offspring from each group.

Delayed type hypersensitivity test (DTH) (Skin test): This test was done according to (Hudson and Hay, 1980).

Detection of serum INF-ɣ:serum INF-Y in mice sera was assessedby using acommercially ELISA kit obtained from kamo biotech CO, LTD. (Korea). The test was carried out according to the manufacture assay protocol.

Detection of (IgG): IgG in mice sera was assessed by using a commercial available ELISA kit obtained from kamo biotech CO, LTD.(Korea). The test was carried out according to the manufacture assay protocol.

Hormonal assay of testosterone: Levels of testosterone hormone in mouse serum were assessed by using a commercially available coulter kit obtained from Immunotech/ Czech Republic (Kit). The result was reported as picogram /milliliter (Pg/ml). The test was carried out according to the manufacture assay protocol.

Semen collection: Semen collection was done according to methods (Bennet and Vickery, 1970).

Sperm morphology: Morphology of sperm was identified according to (Wyrobek *et al*., 1983).

Sperm Number and viability: The viability of sperm was studied according to (Kodama *et al*., 1997).

Determination numbers of stillbirth and offspring fetus:

The numbers of stillbirth and offspring fetuses in all groups were calculated according to the following equation (Sellami *et al*., 2011):-

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, followed by Least Significant difference (LSD) test which were used to determine differences among means of investigating groups. The level of statistical significance was set at ($P < 0.05$) (Snedecor and Cochran, 1989).

RESULTS

Skin test (DTH test)

The results expressed the difference in skin thickness in the immunized group which showed maximum and significant increase at 48 hours (1.8 ±0.07) millmeters after injected the soluble antigen intradermally then beginning to decrease at 72 hours (0.8 ±0.05) millmeters in comparison with control group which showed negative results (table 1).

TABLE1: Difference in Skin thickness (millimeters) of immunized and control groups in DTH test

GROUP	24 hours Mean ± SE Unit (millimeters)	48 hours Mean ± SE Unit (millimeters	72 hours Mean ± SE Unit (millimeters)
G1 (immunized)	0.9 ±0.05 B	1.8 ±0.07 A	0.8 ±0.05 B
G2 (Positive control)	0	0	0
G3 (negative control)	0	0	0

Different capital letter means significant at (P≤ 0.05).

Level of IFNɣ:
The results showed that serum INFY in G1 (immunized group) post immunization were higher (430 ±1.75) Pg/ml than those values in G2 &G3. Thirty days post infection G1 showed the same increase of INFɣ in comparison with G3 while G2 (positive control group) revealed a slight increase (60.50 ±0.4) Pg/ml group (Table: 2).

TABLE 2: Mean values of serum IFNɣ in G1 & G2 post immunization and in G1, G2 & G3 at 30 days post infection:

Groups	IFN ɣ (Mean ± SE) Post 30 days of immunization Unit: Pg/ml		IFN ɣ(Mean ± SE) Post 30 days of infection Unit: Pg/ml
G1(Immunized)	430±1.75 A	Infection	437±1.67 A
G2 (positive)			60.50±0.4 B
G3 (negative)	50.72±0.60 C		51.44±0.70 C

Different capital letter means significant at (P≤ 0.05).

Titers of IgG: The results showed that mean serum IgG titers in G1 were higher (185±1.2) Pg/ml than those values in G2 (30±0.29) Pg/ml at 30 days post infection and G3 group (40±0.25) Pg/ml (Table: 3).

TABLE 3: Mean value of serum IgG titers of immunized and non immunized animals at 60 days post infection:

Groups	(Mean ± SE) Post 30 days of immunization Unit: Pg/ml		(Mean ± SE) Post 30 days of infection Unit: Pg/ml
G1	190±0.90 A		185±1.2 A
G2		Infection	30±0.29 C
G3	41±0.50 B		40±0.25 B

Different capital letter means significant (P≤ 0.05).

Serum Testosterone level: The results expressed that the mean values of serum testosterone in G1 at 30 days post infectionwere (13.3±0.10) higher than those values in G2 (2.8±0.25) and G3 (10.1±0.45) (Table 4).

TABLE4: Mean of serum testosterone post immunization and post infection(Pg/ml):

Groups	(Mean ± SE) Post 30 days of immunization		(Mean ± SE) Post 30 days of infection
G1	12.7±0.90 A		13.3±0.10 B
G2	10±0.20 B	Infection	3.7±0.25 D
G3	10±0.20 B		10.3±0.45 C

Different capital letter means significant at (P≤ 0.05).

Sperm count:
The mean counts of sperms in G1 and G3were (87±3.30; 82±4.50) respectively, which were higher than those values in G2 (5±0.9) at day 60 post infection (Table 5).

TABLE 5: Mean of sperm count post 60 days of infection with *C. pseudotuberculosis*:

Groups	Live range	Dead range	Normal range	Abnormal range
G1	87.00±3.30 A	13±3.30 C	90.00±1.00 A	10±1.00 C
G2	5.00±0.9 C	95.00±0.9 A	5.00±2.20 C	95.00±2.20 A
G3	82.00±4.50 B	18.00±.4.50 AB	85.00±2.40 B	15±2.40 B

Different capital letter means significant at (P≤ 0.05).

Pregnant index: The percentages of pregnant index in normal female mated with infected male with C.pseudotuberculosis were (0%) as compared with those values in group immunized males with CFAg (100%) post 60 days from infection (table 6).

TABLE 6: Mean of pregnant index inthe normal female mice after mating with infected males by *C. pseudotuberculosis* after 60 days post infection:

Groups	Gestation index %	Fertility index %	No. live offspring	
G1	100%	100%	72±1.30	A
G2	0%	0%	0	C
G3	90%	90%	70±1.2	A

Different capital letter means significant at (P≤ 0.05).

Histopathological examination

At thirty days post infection: Section in the testis showed edema that lead to large spaces between deformity of seminiferous tubules(Fig:1) necrosis of all epithelial cells of seminiferous tubules(Fig: 2), in othersections, it was found cellular debris in the lumen of seminiferous tubules(Fig: 3). Sperms were not appearingin the lumen of the epididymis (Fig: 4 and 5), while in normal section are present (fig: 6).

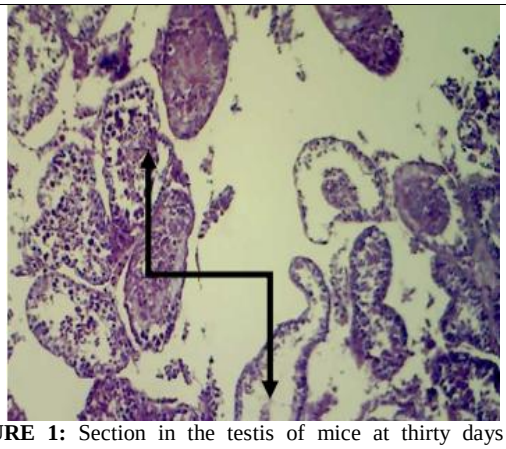

FIGURE 1: Section in the testis of mice at thirty days post infected with *Coronybacteriumpseudotuberculosis* shows edema that lead to large spaces between deformity of seminiferous tubules (↕) (H&E stain 100X).

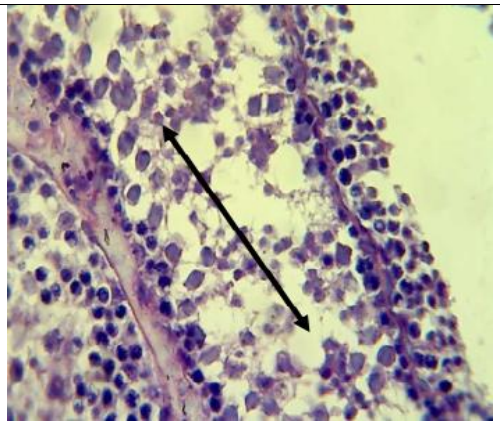

FIGURE 2: Section in the testis of mice at thirty days post infected with *Coronybacterium pseudotuberculosis* shows necrosis of all epithelial cells of seminiferous tubules (↔) (H&E stain 400X).

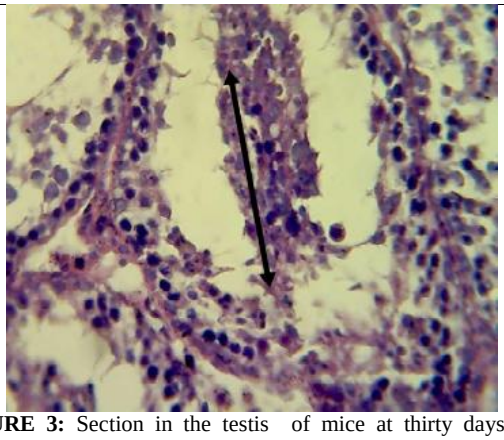

FIGURE 3: Section in the testis of mice at thirty days post infected with *C. pseudotuberculosis* shows cellular debris in the lumen of seminiferous tubules(↔)(H&E stain 400X).

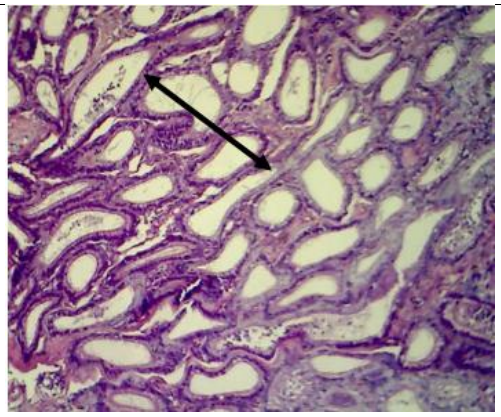

FIGURE 4: Section in the epididymis of mice at thirty days post infected with *C. pseudotuberculosis* shows no sperms in their lumen (↔) (H&E stain 100X).

Immunized animals with CFCAgs

At thirty days post infection: Section in the testis of immunized mice by CFAg at thirty days post infected with *coronybacterium pseudotuberculosis* showed normal arrangement of seminiferous epithelium,leydig cells, sertolo cell,myoid cells, spermatogona A and B, spermatocytes and spermatozoa in the lumen of these tubules(Fig: 7), and there is no any abnormility in the structure ofseminiferous tubules with complete spermitogenesis (Fig: 8) in addition the epididymis lumen were filled with sperms (Fig: 9).

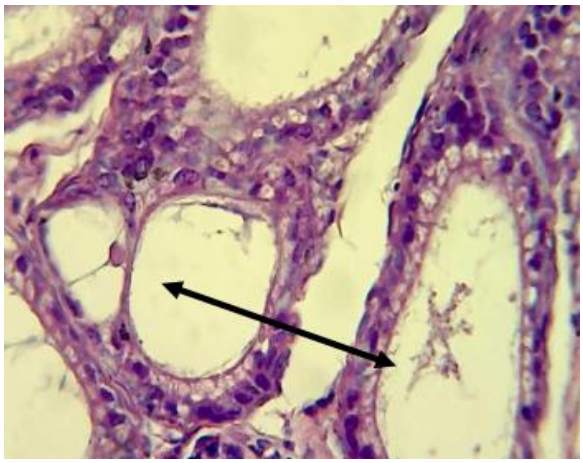

FIGURE 5: Section in the epididymis of mice at thirty days post infected with *C. pseudotuberculosis* shows no sperms in their lumen (➡)(H&E stain 400X).

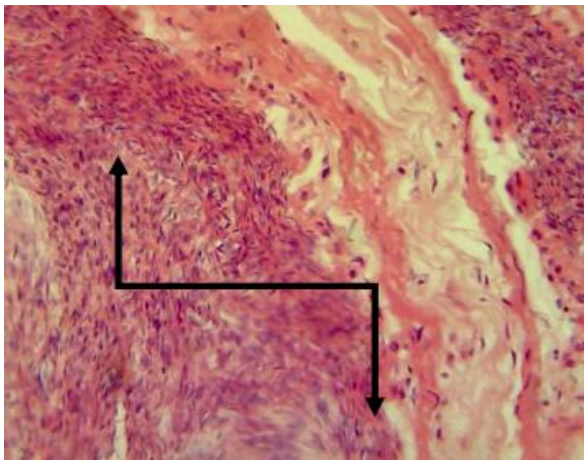

FIGURE 6: Section in the ductull efferentes of epididymis of normal mice shows sperm filled these ductus (⇕) (H&E stain 400X).

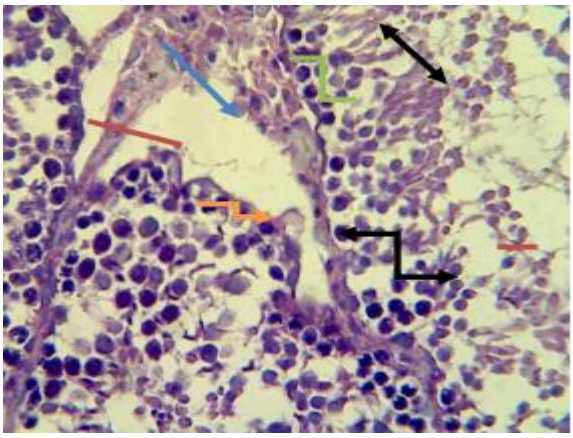

FIGURE 7: Section in the testis of immunized mice by CFAg at thirty days post infected with *C. pseudotuberculosis* shows normal arrangement of seminiferous epithelium,leydig cells (➡),sertolo cell (↪) ,myoid cells (⌐), spermatogona A and B spermatocytes (⇕) and spermatozoa (⇕) in the lumen of these tubules (H&E stain 400X).

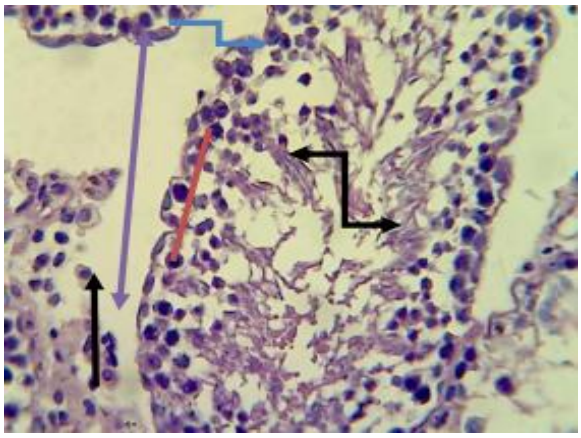

FIGURE 8: Section in the testis of immunized mice by CFAg at sixty days post infected with C*oronybacterium pseudotuberculosis* shows normal arrangement of seminiferous epithelium (➡) sertoli cell (▬) myoid cells (⌐) spermatogona A and B (⇕) spermatocytes () and spermatozoa in the lumen of these tubules (H & E stain 400X).

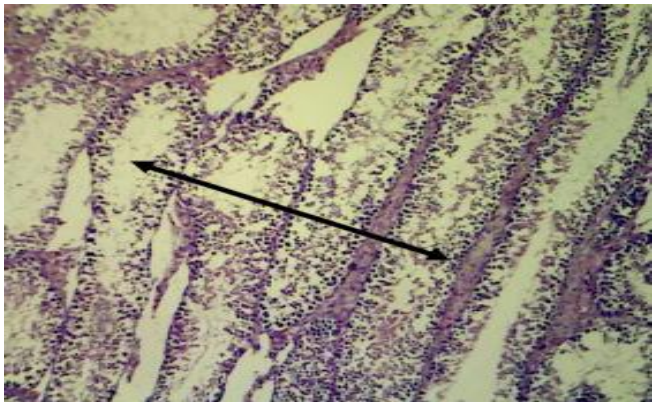

FIGURE 9: Section in the testis of immunized mice by CFAg at sixt days post infected with *C.pseudotuberculosis* shows normal arrangementof seminiferous epithelium with completer spermatogenesis process (⇕) (H&E stain 400X).

DISCUSSION

The current study showed that the Cultural Filtrate Ag stimulated better immune response due to the protein nature of this Ag, this idea was anagreed with (Vale*et al*., 2016) who demonstrated that secreted Ag of this pathogen induced effectiveness spleen cell proliferation as compared with somatic Ag of the pathogen also (Meyer *et al*., 2005) recorded that secreted Ag of *C. psudo tuberculosis* can intense proliferation of lymphocytes in infected goats. The finding of immunization study revealed high levels of serum INF Y in immunized mice, these results may indicate that CFAgs can stimulate CD4 and CD8 T cells to secreted INF Y that was responsible for DTH reaction, this idea was consistent with Tavares-Murta e*t al*. (1996) who investigated that stimulated macrophages and dendritic cells can produce IL12 which stimulated Natural killer cells to produce INFY. Also, these results was an agreed with Vale *et al*. (2016), who demonstrated that, *in vitro*, activated lymphocytes of spleen by secreted and somatic antigens of *C. pseudo tuberculosis* can secrete IFN-γ, IL-4, IL-10, IL-12 and nitric oxide also they recorded high levels of Th1-profile cytokines in mice at 60 days post-inoculation.

The result of INFY examination was agreement with the results of DTH, also these results are inconsistent with high antibody titers in the serum of immunized mice with CFAgs, these results may indicate that secrete Ag of *C. psudo tuberculosis* can stimulated humoral antibodies this idea was inconsistent with Hodgson *et al*. (1993); Paule *et al*. (2004), investigated that CP can produce phospholipase D which considered highly immunogenic component of these pathogens, also the humoral and cellular immune response elicited by CFAg in current study may be represent an attempt to vaccine candidate against CLA disease. The current result showed a low levels of serum testosterone in non-immunized as compared with immunized mic post infection, this may indicate that *C. pseudotuberculosis* infection lead to infertility of infected male, these results was in agreement with khuder*et al*. (2012), who recorded a significant reduced in the levels of testosterone in mice infected with *C. pesudotuberculosis*. Also severe pathological changes in the testis may indicate that these pathogens may be destroyed cells responsible for production of the testosterone and sperm production. Meyer *et al*. (2005) investigated that exotoxin PLD can cause damage cell membrane and prevent protein synthesis that associated with decreasing hormone production particularly testosterone which play essential role in spermatogenesis process. The current study revealed that *C. pseudotuberculosis* infection lead to a high number of dead and abnormal sperms count as compared with those values in the non infected animals, these results may due to direct effects of bacterial exotoxin on the testis and or due to the toxic effects of ROS, these idea was agreement with (Colagar *et al*., 2007) who reported that oxidative stress cause impairment sperm function and decline in quality and quantity of the sperms the present finding was agreement with the result of pregnant gestation of normal female mated with infected males,these observations was inconsistent with Agarwal and Said, (2005); Alkhafajy, (2017) who found that oxidative stress associated with infertility of males. However, the current result revealed that *C. pseudo tuberculosis* cause necrosis and degenerative changes of the seminiferous tubules that lead to destructed of spermatogenic cells and spermatids in addition to atrophy and deformity of the seminiferous tubules, these pathological changes associated with decrease sperm count and no pregnant of normal female that mated by infected male mice infected with C. *pseudotuberculosis*, these result may indicated that these pathogen cause reproductive disorder, may be due to degenerative changes occur in the hypothalamus, pituitary and gonads that lead to decline in concentration of sex hormone particularly testosterones which essential hormone in reproductive process. Also the current study showed a high percentage of pregnant index in normal females that mating with immunized infected males, these findings may indicate that immunized animals by CFCAg can improve fertility in infected animals with *C. Pseudotuberculosis*, this study was considered the first study in Iraq, we do not find any study about the influence of *C. pseudotuberculosis* on a pregnant index of infected animals as well as the role of CFAgs of these pathogens on fertility of animals infected by this pathogen,

CONCLUSION

The current study showed that infected male mice by *C. psudotuberculosis*lead to infertility of the male mice and immunization with CFAgs provide good protective immunity against infection by this pathogen and improvement male mice fertility.

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