Research Article

Effect of *Trypanosoma evansi* on the Efficiency of *Pasteurella multocida* Vaccination

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Abstract | *Trypanosoma evansi* is the most ubiquitous protozoan parasite with a wide geographical distribution. The current study aimed to investigate if *Trypanosoma evansi* induces immunosuppression that may interfere with the development of immunity after vaccination in rats against *Pasteurella multocida*. *T. evansi*-infected and non-infected Wester Albino rats immunized against pasteurellosis with two vaccines; one is commercial, and the other is a formalin-killed vaccine (prepared from a local strain), and subsequently challenged virulent *P. multocida*. The differences in the regulation of IFN-γ, TNF-α, IL12, IL6, and IL10 cytokine levels expression, were assessed as biomarkers of the clinical phase of infections. Results revealed that *T. evansi* infection has a significant influence on the performance of rats, and antibody responses against both vaccines were significantly reduced in *T. evansi*-infected groups compared to controls and others did not contain *T. evansi*. It was accompanied by significant elevations levels in total leukocytic counts, urea, ALT, AST, ALP, and the total protein compared to control values. *T. evansi* developed protection against both vaccines with increases in α-TNF, γ-IFN, IL12, and IL6 levels, and lower in IL-10 levels. The differences in cytokine levels against the two vaccines pointed to a significant role in the immunosuppression of *T. evansi* on vaccines confirmed by severe changes in the lung by different degrees. We concluded *T. evansi* spoils vaccines and produces poor protection in vaccinated rats against *P. multocida* infection.

Keywords | *Trypanosoma evansi*, *Pasteurella multocida*, Vaccine, Rats, Egypt

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INTRODUCTION

Trypanosoma evansi is the most ubiquitous protozoan parasite with a wide geographical distribution (Juyal et al., 2005; Gillingwater et al., 2007). It is highly pathogenic with a high prevalence among several domestic animals and wild mammals and rarely in humans (Hoare, 1972; Joshi et al., 2005), causing a variety of types of pathogenicity (Lun et al., 1992). Respiratory tract diseases included hemorrhagic septicemia, are among the emerging health hazards to domestic animals worldwide and are incurring a considerable loss of life and production (Mochabo et al., 2006; Intisar et al., 2009). *Pasteurella multocida* is a Gram-negative bacillus in the pasteurellaceae family considered a common inhabitant of the upper respiratory tract, which may cause disorders in association with other microorganisms (Wilson and Ho, 2013). Pasteurellosis due to *P. multocida* type A is an acute fatal respiratory disease

in animals that suffer from malnutrition, gastrointestinal parasitism, and exposure to cold (Abubakar et al., 2010). It is associated with hemorrhagic septicemia in camels and is known as Pneumonic Head (Kebede and Gelaye, 2010). Pathogenic trypanosomes induce generalized immunosuppression of humoral antibodies and T-cell mediated immune responses (Stijlemans et al., 2017; Magez et al., 2020). As a result, in the long term, the host immune responses fail and succumb to either the overwhelming parasite load or to secondary infection, consequently leading to the occurrence of trypanosome-induced immunopathology (Eyob and Matios, 2013; Onyilagha and Uzonna, 2019). Several studies undertaken to investigate the influence of *T. evansi* infection on production in livestock suggested that the capacity to mount the immune response against *Theileria*, *Anaplasma* and *Babesia* may be immunosuppressed in *T. evansi* infected camel. It demonstrated that *T. evansi* infection lowers the immune responsiveness of camels to concurrent immunizations (Holland et al., 2003; El-Naga and Barghash, 2016). Also, *T. evansi* infection might interfere with the protective immunity development upon heterologous vaccinations causing poor protection of *Pasteurella*-vaccinated buffaloes in *T. evansi*-endemic areas of Vietnam (Holland et al., 2001). The generation of suppressor T-cells and macrophages altered antigen handling and presentation are mechanisms involved in trypanosome-mediated immunosuppression (Sileghem and Flynn, 1994). Phospholipases, neuraminidases, and proteases are trypanosome enzymes involved in membrane fluidity and causes cellular damage (Fung et al., 2007).

Likewise, experimental infections in buffalo and pigs have shown reduced cellular, in addition to humoral responses after vaccination against classical swine fever and *P. multocida* in *T. evansi* infected animals compared to uninfected animals (Holland et al., 2001, 2003). It indicated by a lost capacity to mount humoral and cell-mediated immune responses against heterologous antigens in cattle and buffaloes (Desquesnes et al., 2013). It would be responsible for the failure of the vaccination campaigns against foot and mouth disease (FMD) and hemorrhagic septicemia (Singla et al., 2010, 2012; Çokçalışkan et al., 2019). It also affects immune responses in sheep by delaying and depressing the number of lymphoblasts induced by *P. haemolytica* vaccine administration (Onah et al., 1997), as well as pigs by interfering with their immune response to the classical swine fever (CSF) vaccine (Holland et al., 2003). In Egypt, no vaccines are available against *P. multocida* in camels, but one commercial vaccine (A: Pneumobac® vaccine), is used for sheep and cattle vaccination. The present study aimed to investigate if *T. evansi* induced immunosuppression in experimental rats may interfere with the development of immunity after vaccination against *P. multocida* infection and confirmed the result with the histopathology of the lung. Also, to evaluate and compare the protective efficacies of *P. multocida* vaccines, the formalin-killed whole-cell antigen (FKA) prepared from local *P. multocida* strain isolated from camel, and the commercial vaccine (Pneumo-bac®, VSVRI).

MATERIALS AND METHODS

Ethical approval

The current experiment was conducted in compliance with internationally accepted principles of the European Directive 2010/63/EU for laboratory animal use, the Declaration of Helsinki (1964), and the National Institutes of Health guide (NIH Publications No. 8023 revised 1978). Also, it was following International Guiding for Biomedical Research Involving Animals, 2012.

T. evansi strain

It was obtained from a naturally infected camel at Ras-Sudr (South-Sinai, Egypt), and maintained in the laboratory by the continuous passage in white Swiss mice, then purified, sequenced, and BLASTn with the existing sequence of *T. evansi* in the GenBank database and identified with an accession number of MZ198121. Parasitemia level was calculated daily until giving parasitemia of approximately 10^5 Trypanosoma/ml, according to Hoare (1972).

Bacterial strain

P. multocida strain was isolated from a pneumonic lung obtained from a freshly slaughtered camel from South-East Egypt, according to Jakeen et al. (2016). It is identified and referenced in the GenBank database under the accession number MW925053.

Vaccines

Two vaccines against *P. multocida* are evaluated in the present study; one commercial for sheep and goats (Pneumobac, VSVRI) (A) purchased from Veterinary Serum and Vaccine Research Institute (Abbasia, Cairo, Egypt). Another one is formalin-killed Vaccine (B) from *P. multocida* local strain obtained from camel and prepared according to Ruzauskas (2005) and Roetzera et al. (2017).

Bacterial infection challenge

P. multocida was grown on dextrose starch agar plates overnight at 37°C. The cells were harvested in 0.01 M phosphate-buffered saline (PBS), centrifuged, washed twice in PBS, and diluted to a final concentration of 3.6×10^{10}/ml, according to Ahmed et al. (2016).

Experimental animals and design

Eighty male Swiss Albino Wister rats weighing 200 g from Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt, were kept in well-ventilated plastic cages. They

were exposed to 12 hours light and dark cycles, watered and fed with pellets and fresh vegetables throughout the experimental period. The animals were allowed 10-days of acclimatization before the experiment. They were classified into eight groups of ten rats per cage and subjected to treatments, as illustrated in detail in Table 1. All rats were observed daily for signs and symptoms.

Biological analysis

Blood samples were collected after 1st immunization for haematological examination at 0, 15, 30, 45, 60, 75, and 90 days, and sera were frozen at –20 °C until used. The haematological investigation and biochemical analyses: For glucose, bilirubin, uric acid, creatinine, total protein, albumin, liver enzyme alanine transferase (ALT), aspartate transferase (AST), and alkaline phosphatase (ALP) were estimated following standard methods, using commercial test kits (Spectrum, Egypt). Tissue specimens from lungs were fixed in 10% neutral buffer formalin solution. After complete fixation, they were dehydrated in ethyl alcohol, embedded in paraffin, sectioned at 5µm thicknesses, stained with Hematoxylin-Eosin stain for general histopathological examination, according to Bancroft and Stevens (1966).

Vaccine efficiency

The antibody productions were evaluated using optical density as an indicator for the efficiency of the tested vaccines against *P. multocida* to generate an immune response. Sera samples were assayed for antibodies against *P. multocida* by enzyme-linked immune-sorbent assay (ELISA). The plates were read at 405 nm spectrophotometrically using an ELISA reader (BioTek ELX800, with software Gen52.00).

Cytokine determination

ELISA was used to determine IL6, IL10, IL12, TNF-α, and IFN-γ titers at 0, 15, 30, 45, 60, 75, and 90 days in the sera of the immunized rats. The levels of tumour necrosis factor (TNF-α) were quantified by sandwich ELISA, with Ray BroR Rat Kit TNF-α (KOMA BIOTECH INC). IBL Rat ELISA set IL6, IL10, IL12, and Interferon-Gamma (IFN-γ) (Immunological Laboratories Inc., Meme Minneapolis, Minnesota, USA), following manufacturer instructions.

Data analysis

The absorbance value of ELISA for evaluation of antibody levels and detection of cytokines were statistically analyzed for difference among groups by performing ANOVA. A *P*-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Clinical observation

In the present study, no death in (N) rats, contrary to rats in (T) that died on the third week and after 36 hours in (P). On the other hand, rats in (TP) gave clues of dullness after 24 hours, and three rats died then all rats after 48 hours. Concerning groups (AP, BP); no death but after challenge in group AP (five rats died), and three died from group BP. In the same experiment, rats in groups (TPA, TPB) were regularly examined every hour for the appearance of symptoms after the challenge. After 24 hours, the signs of dullness developed compared to the healthy ones, followed

Table 1: The status of the experimental groups before and after vaccination.

Group	Code	Status
G1	N	Injected subcutaneously (SC) with 2 mL of sterile PBS and kept as -Ve group.
G2	T	Infected intraperitoneally (IP) with 0.1 mL of blood from infected mice containing 3 x 10^5 trypanosomes (+Ve *T. evansi*).
G3	P	IP injected with *P. multocida* 0.5 mL of 3.6 × 10^{10} bacterial cells/dose (+Ve *P. multocida*).
G4	TP	IP route infected with *P. multocida* of approximately 3.6 × 10^{10} bacterial cells/dose at the same time 100 µl infected blood with *T. evansi*/ rat.
G5	AP	Vaccinated (S/C) with two doses of commercial vaccine A, After three weeks booster dose was administered then challenge (I/P) with live *P. multocida* specific strain at 1×10^{-3}dilution.
G6	BP	Vaccinated (S/C) with two doses of vaccine B, adjuvant with 0.5% aluminium potassium sulfate prepared from a local strain isolated from camel against *P. multocida* at 4 × 10^9 bacterial cells/ dose followed by a booster dose which was given three weeks after the first one, then challenge (I/P) with live *P. multocida* specific strain at 1×10^{-3}dilution.
G7	TPA	Contained infected rats with 0.1 mL of 3x10^5 *T. evansi*, then vaccinated (S/C) with two doses of vaccine A; the booster dose of commercial vaccine was three weeks after the first dose, then challenge with 0.5 ml *P. multocida* at 4 ×10^9 bacterial cells/ dose.
G8	TPB	Infected rats with 0.1 mL of 3x 10^5 *T. evansi* were vaccinated (S/C) with two doses of vaccine B, followed by the booster dose at three weeks after the first dose, then challenge with 0.5 ml *P. multocida* at 4 × 10^9 bacterial cells/ dose.

by the death of all rats after 72 hours. Dead rats had gross lesions and severed congestion with an abscess in all internal organs.

The effect of *T. evansi* on rats inoculated with single *P. multocida* and with that vaccinated against pasteurellosis was studied. Haematological analysis and clinic-biochemical results of the infected and treated rats revealed a significant difference ($p \leq 0.05$) between the groups, harboured *T. evansi* infection and those only vaccinated and challenged with *P. multocida* groups until the end of the experimental period (Figure 1). Whereas, *T. evansi* delayed and depressed the increases in HB and RBCs output in the single infected group with *T. evansi* (T), mixed infections (TP), and in vaccinated groups (TPA, TPB) in contrary to total WBCs levels that elevated in these groups. In the non-infected group (N), the total cellular output peaked at more than two times the *T. evansi* infection values after primary infection but smaller after injection with *P. multocida*.

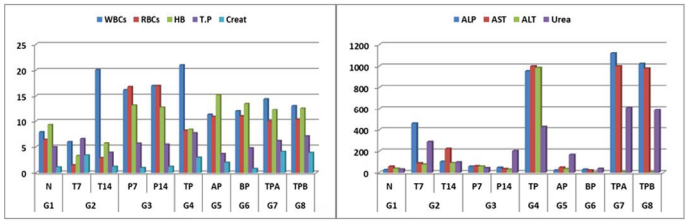

Figure 1: The clinic-biochemical in single and mixed infection, and after vaccinated and challenged with *P. multocida* in the groups of rats.

On the other hand, the output of biochemical tests revealed significant differences between groups with and without trypanosomosis. It peaked at vaccinated groups with *T. evansi* (TPA, TPB) compared to the pre-vaccination values without *T. evansi* (AP, BP) and more than a single infection with *T. evansi* (T). It was three times more in single *T. evansi* and 5-30 times in *P. multocida* vaccinated one (P). In response to the infection with *T. evansi*, the levels of Urea, Creatinine, ALT, AST, ALP, and T. Protein showed sharply elevation in *T. evansi* infected groups (T), (TP), (TPA), and (TPB) compared to *P. multocida* and vaccinated groups (P, AP, and BP).

Evaluation of the two vaccines using ELISA

HS-specific antibody responses

The effects of *T. evansi* infection on the antibody titers against the two vaccines are illustrated in Figure 2. Antibodies following primary vaccination with a single B-vaccine were detected as early as day 7 (0.312±0.00). After the first week, a higher titer in the BP group (0.758±0.006) than AP group (0.527±0.014), and very low titers at TPB group (0.307±0.004) and TPA group (0.359±0.008) were patent. The highest antibody titers after 21 days reached 0.861±0.009 was at BP group than 0.598±0.033 at AP group, whereas the lowest titers were recorded at (0.290±0.023) and (0.355±0.010) of TPB and TPA groups, respectively.

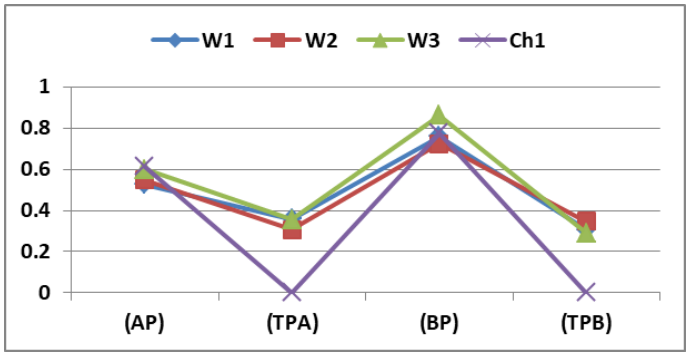

Figure 2: Specific antibodies responses against the two vaccines and after challenge in presence and absence of *T. evansi*.

Protective efficacy of vaccines

The results of the protective efficacy of vaccines were evaluated in rats after a challenge with *P. multocida* specific strain. Animals were challenged on day 28 (2 weeks after second and final immunization) with *P. multocida* strain at a dose of 1.5×10^7 CFU/mL (IP) and observed for 14 days post-challenge. The results showed that the mortality rate in *T. evansi* plus A-vaccine (TPA) and *T. evansi* plus B-vaccine (TPB) (100%) was higher than that in BP which vaccinated only with two doses of B-vaccine (30%) and AP which vaccinated only with two doses of A-vaccine (50%) in an inactivated oil adjutant polyvalent *Pasteurella* vaccine.

Changes in serum cytokines titres

To investigate the cellular immune response of different immunogens: Th1 cytokines (IL12, IFN-γ, TNF-α) and Th2 cytokines (IL6 and IL10) levels were determined in the eight groups (Figure 3). Also, to correlate the clinical findings with the immunological response observed during vaccination against *P. multocida* in *T. evansi* infected rats. Besides, to identify potential immunological pathogenesis markers that can stimulate antibody production circulating in the present study. The values of TNF-α and IL6 were consistently higher in all groups than in the negative control group and, no differences between commercial and local vaccinated groups. IL12 level in the B group was extremely statistically significantly higher than that in other immunized and control groups (P=0.0001). The present results also showed that sera produced higher levels of TNF-α, IL12, IFN-γ in all groups (except A, B). There is a correlation with no difference between TNF-α, IL12, IFN-γ levels, and IL6 levels that belong to Th2 cytokines, with IL10, which behaved the opposite. However, IL10 produced with high levels only in vaccinated groups than lower levels in other groups.

Histopathological examination

The changes were seen microscopically in the lungs and were consistent with trypanosome infection. The results of the histopathological abnormalities in the lungs are shown in Figure 4. It was more severe and evident in vaccinated groups with trypanosome infection than others. All groups were associated with no weight loss in rats compared to the *T. evansi* infected groups. The lung of G2 (T) showed peribronchitis with peribronchial mononuclear cell infiltrations (arrowhead) and peribronchial congested blood vessel (arrow) (H and E X 200). Whereas in G3 (P), the lung shows broncho interstitial pneumonia representing by bronchitis (arrowhead) and severe interstitial inflammatory reaction, congestion and leucocytic cell infiltrations (H and E X 200). Mixed infections in G4 (PT) causes change vasculitis representing by congestion (arrow) and thick wall muscle layer (arrowhead) (H and E X 200). Sections of lungs in vaccinated groups G5 (AP) and G6 (BP) showed less diffuse interstitial pneumonia with marked interstitial thickening (arrowhead) and mononuclear cells infiltration (arrow) (H and E X 400). In contrast, lungs in infected and vaccinated groups: G7 (TPA) and G8 (TPB) show interstitial pneumonia representing by severe interstitial inflammatory reaction; congestion, oedema (arrowhead), alveolar haemorrhage, and leucocytic cell infiltrations (arrow) (H and E X 100).

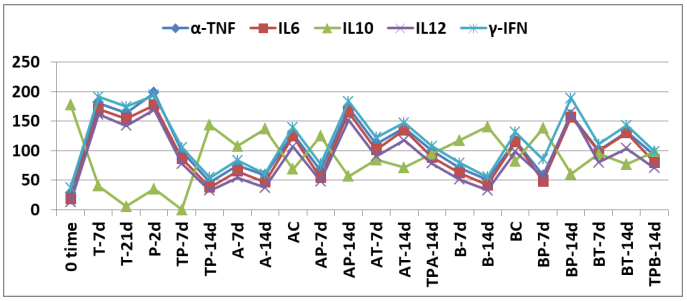

Figure 3: Development of TNF- α, IL6, IL10, IL12, and IFN-γ quantification by ELISA in the sera of rats infected with *T. evansi*/ or *P. multocida* and vaccinated against Pasteurellosis along with the experiment. d= day, ac= after challenge.

Trypanosomosis is a disease affecting the immune system of the host animal (Singla and Juyal, 1992; Lutje and Mertens, 1995). Although the immune system is designed to protect a host from pathogens, it can sometimes be overwhelmed, respond inappropriately, or result in immune-mediated disease with clinical signs or succumbs to either the overwhelming parasite load or secondary infection (Stijlemans et al., 2007; Gupta et al., 2009). *T. evansi* is occasionally accompanied by *P. multocida* when animals are weakened due to stress caused by a parasite, viral and bacterial infection, or change in climatic conditions (Kebede and Gelaye, 2010; Singla et al., 2015; ElNaga and Barghash, 2016). *T. evansi* as purely extracellular parasites survives, multiply, and differentiates in extracellular fluids of the mammalian host, including the aggressive vascular environment. Thus, these parasites are permanently confronted with the multiple components of the host immune system ranging from innate to adaptive immune defences (Magez et al., 2020).

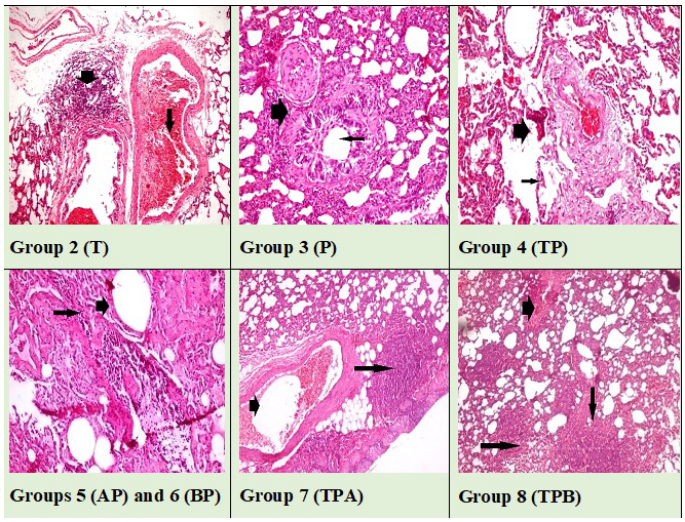

Figure 4: Histopathological changes in the lungs of *T. evansi* experimentally infected groups of rats, with and without vaccination against *P. multocida*.

To determine whether *T. evansi* induces immunosuppression or may induce immunosuppression, the present investigation was intended to characterize the clinical phases of trypanosomosis in experimentally infected rats, with the difference in the regulation of cytokine expression. The diagnostic potential of IFN-γ, TNF-α, IL12, IL6, and IL10 as biomarkers of the clinical phase of infections compared to parasitological and biochemical levels was studied. Analysis of the results showed an imbalanced immune response in the eight groups, suggesting that an uncontrolled immune response is associated with the present *T. evansi*. In some helminthic infections (Finkelman et al., 1991; Lange et al., 1994), the Th1 (produce IL12, IFN-γ) and Th2 (express IL6, IL10) cytokine secretion patterns down-regulate each other (Pearce et al., 1991). But the co-expression of IFN-γ, TNF-α, IL12, IL6 at high levels in contrary to IL10 levels in the present study suggests that the immune response may be due to a complex mixture of antigens in the blood of rats which probably contain distinct epitopes for the two pathogens as reported by McManus and Bryant (1995). Besides, secondary infections cause more severe complications. But in the present study, the immunosuppressive cytokine, IL10, is overexpressed. It inhibited the production of proinflammatory cytokines (IL6 and TNF-α) as reported by Kirchberger et al. (2007). IL6 is produced by antigen-presenting cells such as macrophages, dendritic cells, and B cells. Also, it is produced by non-hematopoietic cells as fibroblasts in response to external stimuli such as TNF-α,

platelet-derived growth factor, or microbial components. IL6 receptors are only present on directly activated B-cells which increase IgM and IgG antibodies (Jones, 2005). Trypanosome killing is assumed to occur via the induction of classically activated macrophages that produce high levels of inflammatory compounds such as tumour necrosis factor α (TNF-α) and reactive oxygen intermediates (Vincendeau and Boutwill, 2005).

Despite several previous studies on *T. evansi* infection that showed a correlation between the intensity of inflammation and the level of parasitemia, the circulating trypanosomes in some studies failed to show that because the immune response was directed against both parasites and self-antigens (Soares and Santos, 1999). However, these pathogens defy humoral immunity through a subtle mechanism of antigenic variation (Mosa, 2020). The antibodies directed against the specific surface-exposed epitopes of the VSG coat opsonize the parasites. Besides, the immune complexes are efficiently phagocytosed and destroyed, mainly in the liver, by the macrophages (Barghash, 2019). It may be responsible for the high levels of production in the infected groups with *T. evansi* (single, mixed infection, and that vaccinated against *P. multocida*). Also, the VSG may be released from the dead trypanosomes activated macrophages to secrete proinflammatory molecules like TNF-α, IL6, IL12, and IL10, as a response of the host immune system. It is involved in the first peak of parasitemia by the toxic nature of TNF-α for both the host cell and the parasite (Finkelman et al., 1991). A previous study has highlighted the capability of *T. evansi* in pro-inflammation or anti-inflammation activities (El Sayed et al., 2017). The elevated IL10 levels are correlated with poor survival (Kubatzky, 2012). Among other factors such as lipopolysaccharide, outer membrane proteins and their capsule, the protein toxin (PMT) of *P. multocida* is an important virulence factor that determined the immunological response of the host's immune system in agreement with Stijlemans et al. (2018).

The present results showed a correlation in analysis between inflammatory cytokine production in commercial and locally prepared vaccines against *P. multocida*. It also showed the displayed negative correlation between IFN-γ and IL-10 levels in serum and positive correlation with other cytokines indicates a controlled inflammatory response in infected and vaccinated groups. The elevated levels of IL-10 that produced *in vivo* experimental infection were correlated with poor survival in rats and accompanied with severe pathological changes which increased in rats of TPA and TPB vaccine groups. It is in agreement with primary and secondary infections that elicited similar responses included elevated levels of IL10. Notably, various studies have shown that polyclonal B cell activation, generation of suppressor T-cells and macrophages, and altered antigen handling and presentation are all mechanisms that could be involved in trypanosome-mediated immunosuppression (Sileghem and Flynn, 1994). In the present study, cytokines responsible for B-cell activation and T-cell suppression were released.

During trypanosome infections; TNF-α secreted by classically activated macrophages is involved in both parasitemia control and infection-associated pathologies like anaemia, organ lesion, and fever. These findings support the hypothesis that a lack of efficient regulation between IFN-γ and IL10 cytokine productions associated with immune-regulation of effector responses may lead to immunopathogenesis as reported before (Raes et al., 2002). In the current study, lymph nodes and spleen were remarkably reactive in fulminated parasitemia. It may account for the generalized lymphoid tissue hyperplasia characteristic of *T. evansi* infections, while the immune system became depleted of lymphoid cells in the late stages, in agreement with Ghaffar et al. (2016).

Regarding histopathological studies, lungs in experimental single and mixed infections and after vaccination against *P. multocida* in the presence of *T. evansi* in rats appeared oedema, congestion, emphysema, and infiltration with inflammatory cells accompanied with alveolar haemorrhage. These changes were due to vasodilatation and exudation mainly caused by the inflammatory response. Also, it may be due to an immune complex deposition and complement cascade reaction, as reported by Abd El-Baky and Salem (2011). These changes in the infected lung tissue were similar to those seen in the lung of various infected animals with *T. evansi* (Biswas et al., 2001; Otto et al., 2010; Barghash et al., 2018; Barghash, 2021).

In summary, the present study revealed the possible use of cytokines as immunological markers of clinical evolution in the sera of rats' harbouring trypanosomosis. It assessed the levels of antibodies administered by the local prepared and commercial vaccines against Pasteurellosis (alone or accompanied with *T. evansi*) using ELISA assay. The immune response depended on gross and microscopic lesion scoring of the lung, mortality, and haemo-biochemical changes. Cytokines levels were significantly reduced in *T. evansi* infected animals and responded against *P. multocida*. The inductive capacity to mount immune responses against the heterologous antigens was suppressed in *T. evansi*-infected animals. Consequently, *T. evansi* infection interfered with that or might inhibit total cytokines may play a role in the induction of immunosuppression by the parasite. We advise that the treatment with anti-trypanosomal drugs may help in reducing the failure of vaccination programs.

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NOVELTY STATEMENT

This study has examined the effect of *T. evansi* on vaccination against Pasteurellosis, besides the efficiency of imported commercial vaccine compared to the local *in vitro* prepared one. It also supported the possible use of cytokines as immunological markers.

AUTHOR'S CONTRIBUTION

SB had the idea, planned, designed the work, prepared *T. evansi* strain, and analyzed the clinical results. AH has prepared *P. multocida* strain, formol-killed vaccine, and challenge. Except that, this work is a product of the two members who have contributed in equal degrees to the analytical methods used, to the research concept, and the experimental infection. The two authors have contributed to writing and approved the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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