Isolation and propagation of an Egyptian Theileria annulata infected cell line and evaluation of its use as a vaccine to protect cattle against field challenge

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Abstract

Tropical theileriosis is an important protozoan tick-borne disease of cattle. Vaccination using attenuated schizont cell lines is one of the methods used for controlling the disease. This study describes the production of an attenuated *T. annulata* cell line from Egypt and an evaluation of its use as a vaccine to protect calves against clinical disease upon field challenge. Two groups of exotic and crossbred male calves were divided into vaccinated and control groups. Vaccinated groups were inoculated with 4 ml (1 x 10^6 cells/ml) of the attenuated cell line. Three weeks after vaccination, calves of both groups were transported to the New Valley governorate where they were kept under field conditions and exposed to natural *T. annulata* challenge. All animals of the control group showed severe clinical signs and died despite treatment with buparvaquone after a severe drop in the packed cell volume (PCV). Animals of the vaccinated group became seropositive without developing serious clinical signs except for the transient fever. Post-mortem examinations revealed enlarged and fragile lymph nodes, spleen, and liver with necrosis and hemorrhages. These findings indicate that the Egyptian attenuated cell line was successful in protecting both exotic and crossbred animals against tropical theileriosis under field conditions.

Introduction

Bovine theileriosis caused by *Theileria annulata* is an important disease of cattle in subtropical and tropical regions where vector ticks of the genus Hyalomma occur [1–3]. The infection is transmitted from ticks to their bovine host through tick saliva containing *T. annulata* sporozoites. The sporozoites enter the host's bloodstream and travel to the lymph nodes. Here, they infect the lymphocytes and transformed through asexual reproduction to the schizont stage. The schizonts divide rapidly, producing many merozoites. The schizont can regulate the cell function and cell programmed death through secretion of proteins directly into the cell cytoplasm, affecting cell signaling and function. This results in cell transformation, making the host cell immortal so that it proliferates continuously. The merozoites then infect other lymphocytes and continue the cycle of asexual reproduction. The merozoites are released into the bloodstream and infect erythrocytes (red blood cells). Inside the erythrocytes, the merozoites undergo further development and divide, producing more merozoites. This stage is responsible for the clinical signs of tropical theileriosis. Some of the merozoites differentiate into male and female gametocytes and when the tick feeds on an infected animal, it ingests the gametocytes, which then undergo sexual reproduction in the tick's gut. The fertilized eggs develop into sporozoites, which migrate to the tick's salivary glands, ready to infect the next host [4,5].

The parasite distribution into the daughter cells is accompanied by a tight association with the host cell mitotic apparatus [6]. *Ta*-transformed leucocytes lose their virulence upon long term passage and are used as live vaccines in endemic countries [7,8]. In Egypt, clinical cases of theileriosis have been reported in different breeds of cattle and buffaloes (*Bubalus bubalis*) all over the Nile valley and other localities such as the Delta region of the Nile valley, Upper Egypt and the Egyptian oasis [9,10]. Early recognition of clinical cases plays an important role in the treatment and control of the disease [11,12]. During the acute phase when relatively high parasitemia are found, it can be easily diagnosed by the use of Giemsa stained blood and lymph node aspiration smears [2,13,14]. During the late chronic phase or carrier stage, the parasitemia drops to microscopically undetectable levels and the diagnosis mainly depends on serological and molecular assays [15,16]. In Egypt, there is a lack of reliable integrated control programs that include accurate diagnosis, tick control, chemotherapy and vaccination, which has hampered control of the disease [17]. On the other hand, successful vaccination using live attenuated schizont-infected cell lines has been reported in many countries in North Africa, the Middle East and South Asia [11,12,18,19]. However, concerns that parasite isolates might not be identical in their antigenic composition or virulence have led to the propagation of local *T. annulata*-infected cell lines and assessment of their efficacy under experimental and field challenge in each country or region. This also triggered the current study, which aimed to produce a local vaccine for Egypt by the isolation and attenuation of infected peripheral blood leukocytes (PBMC) from naturally infected cattle, followed by the evaluation of its potential as a vaccine to protect cattle against *T. annulata* exposure in the field.

Results

Cell lines

Theileria annulata schizont-infected cell line was prepared from leukocytes isolated from both heparinized blood and lymph node aspirates. DNA extracts of the cultures were tested by 18S rRNA and RLB assays followed by sequencing and were positive only for *T. annulata* and not for other tick-borne pathogens. The partial 18S rRNA gene sequences of the isolate were submitted to GenBank and can be retrieved using accession numbers MN704769 and MN704770, respectively.

Vaccination trial

All animals tested negative for *T. annulata* infection by 18S rRNA and Tams1 PCR and TaSp ELISA before vaccination. After vaccination and before field challenge, only animals of the vaccinated group became PCR-positive two weeks after vaccination, and TaSp antibodies were

detected 4–5 weeks post vaccination. None of the animals developed clinical disease. Piroplasm or schizont stages were not observed microscopically in any of the vaccinated animals. Within several hours of the start of the field challenge all animals became infested with ticks. The ticks mainly attached to the scrotum, dewlap, inner side of the thighs and tail. The first clinical signs indicative for *T. annulata* infections were observed after 5 to 12 days post tick infestation. These signs included fever with body temperatures of up to 41.5°C, ocular discharge, enlargement of superficial lymph nodes, in particular of the prescapular and prefemoral lymph nodes, various degrees of respiratory signs including nasal discharge and coughing, pale mucous membranes, jaundice, and diarrhea. The clinical signs were more severe in the control group compared to the vaccinated group. Animals in the control group became anorexic and died despite treatment with buparvaquone at the recommended dose of 2.5 mg/kg body weight. The treatment was applied after animals had persistent fever for two days. The PCV ranged from 26.5–40% before challenge in both vaccinated and control groups and dropped after field challenge to 18–30% in vaccinated animals and 3–34% in the control group (Fig. 1, Table 1,2). The TaSp antibodies increased in all animals. The antibodies' titers before and after challenge being statistically different in the vaccinated group when compared to the control group (Fig. 2). Animals of control groups became positive by both Tams-1 and 18S rRNA PCR within ten days post challenge, and this was confirmed by sequencing. The obtained sequences were identical, and we submitted one of them as an example to the GenBank and it is available under accession number MN7047671.

Table 1

Follow up Parameters before and after challenge in Exotic breed calves including start of fever (day post infection), maximum temperature (of fever), percentage of PCV before challenge and lowest after challenge. Additionally, the maximum parasitemia, treated or not and fate are displayed.

Animal ID	Group	Breed	Start of fever	Maximum temperature	PCV / Hematocrit before challenge	Lowest PCV observed/ Hematocrit	Maximum parasitemia	Treatment	Fate
6947	Vaccinated	Exotic	Day 10	39.9°C (day 10)	27.9%	19%	Post. (0.0004)	No Treatment	Survived
6881	Vaccinated	Exotic	Day 9	40°C (day 10)	28.2%	20%	Post. (0.0008)	No Treatment	Survived
6909	Vaccinated	Exotic	Day 8	40.1°C (day 10)	32.4%	20%	Post. (0.00064)	No Treatment	Survived
1V	Vaccinated	Exotic	Day 7	39.8°C (day 7)	32.8%	22%	Post. (0.0004)	No Treatment	Survived
2V	Vaccinated	Exotic	Day 10	39.9°C (day 10)	33.6%	22%	Pos. (0.0004)	No Treatment	Survived
3V	Vaccinated	Exotic	Day 10	39.9°C (day 10)	28.1%	21%	Post. (0.0008)	No Treatment	Survived
4V	Vaccinated	Exotic	Day 10	40.5°C (day 12)	32%	21%	Pos. (0.00056)	No Treatment	Survived
5V	Vaccinated	Exotic	Day 11	40°C (day 11)	33. 8%	20%	Post. (0.0004)	No Treatment	Survived
6V	Vaccinated	Exotic	Day 10	39.6°C (day 10)	26.5%	21%	Post. (0.00064)	No Treatment	Survived
7V	Vaccinated	Exotic	Day 10	40°C (day 10)	33%	18%	Post. (0.0004)	No Treatment	Survived
8V	Vaccinated	Exotic	Day 9	40.2°C (day 10)	37%	23%	Post. (0.0008)	No Treatment	Survived
9V	Vaccinated	Exotic	Day 9	40.7C (day 10)	40%	19%	Post. (0.0008)	No Treatment	Survived
6907C	Control	Exotic	Day 7	41.5°C (day 8)	28.5%	5%	Pos. (0.0016)	Treated	Died at day 28
6911C	Control	Exotic	Day 7	41°C (day 8)	28.5%	7%	Pos. (0.0012)	Treated	Died at day 21
6913C	Control	Exotic	Day 10	41.5°C (day 10)	28.2%	3%	Pos. (0.00128)	Treated	Died at day 56
1C	Control	Exotic	Day 8	41°C (day 8)	36%	5%	Pos. (0.0016)	Treated	Died day 70
2C	Control	Exotic	Day 7	41°C (day 8)	34%	7%	Pos. (0.0016)	Treated	Died at day 35
3C	Control	Exotic	Day 7	40.5°C (day 8)	33%	8%	Pos. (0.00144)	Treated	Died at day 28
4C	Control	Exotic	Day 7	41°C (day 7)	35%	5%	Pos. (0.00152)	Treated	Died at day 28
5C	Control	Exotic	Day 7	41°C (day 7)	33.5%	7%	Pos. (0.0016)	Treated	Died at day 21
6C	Control	Exotic	Day 7	41°C (day 10)	36.2%	13%	Pos. (0.002)	Treated	Died at day 28
7C	Control	Exotic	Day 7	40°C (day 10)	29.5%	5%	Pos. (0.037)	Treated	Died at day 28
8C	Control	Exotic	Day 5	40.6°C (day 8)	33%	7%	Pos. (0.002)	Treated	Died at day 21

Animal ID	Group	Breed	Start of fever	Maximum temperature	PCV / Hematocrit before challenge	Lowest PCV observed/ Hematocrit	Maximum parasitemia	Treatment	Fate
6947	Vaccinated	Exotic	Day 10	39.9°C (day 10)	27.9%	19%	Post. (0.0004)	No Treatment	Survived
6881	Vaccinated	Exotic	Day 9	40°C (day 10)	28.2%	20%	Post. (0.0008)	No Treatment	Survived
9C	Control	Exotic	Day 8	40°C (day 8)	28.2%	13%	Pos. (0.002)	Treated	Died at day 28

Table 2

Follow up Parameters before and after challenge in Crossbreed calves including start of fever (day post infection), maximum temperature (of fever), percentage of PCV before challenge and lowest after challenge. Additionally, the maximum parasitemia, treated or not and fate are displayed.

Animal ID	Group	Breed	Start of fever (day after tick infestation)	Maximum temperature (°C)	PCV / Hematocrit before Challenge	Lowest PCV observed/ Hematocrit	Maximum parasitemia	Treatment	Fate
256255	Vaccinated	Crossbreed	Day 6	39.9°C (Day 7)	40%	20,5%	Post. (0.0004)	No treatment	Survived
256310	Vaccinated	Crossbreed	Day 6	40°C (Day 10)	34.2%	19%	Post. (0.0004)	No treatment	Survived
239967	Vaccinated	Crossbreed	Day 5	40.1°C (Day 11)	31.1%	20%	Post. (0.00048)	No treatment	Survived
251243	Vaccinated	Crossbreed	Day 8	39.8°C (Day 7)	30.1%	20%	Post. (0.0004)	No treatment	Survived
10V	Vaccinated	Crossbreed	Day 11	39.9°C (Day 7)	33%	20%	Post. (0.0004)	No treatment	Survived
11V	Vaccinated	Crossbreed	Day 10	39.9°C (Day 7)	34%	24%	Post. (0.0008)	No treatment	Survived
12V	Vaccinated	Crossbreed	Day 8	40.5°C (Day 9)	33%	25%	Post. (0.0008)	No treatment	Survived
13V	Vaccinated	Crossbreed	Day 7	40°C (Day 8)	37%	29%	Post. (0.0008)	No treatment	Survived
14V	Vaccinated	Crossbreed	Day 8	39.6°C (Day 10)	39%	21%	Post. (0.00064)	No treatment	Survived
15V	Vaccinated	Crossbreed	Day 9	40°C (Day 8)	36%	30%	Post. (0.0008)	No treatment	Survived
16V	Vaccinated	Crossbreed	Day 10	40.2°C (Day 13)	37%	21%	Post (0.00056)	No treatment	Survived
17V	Vaccinated	Crossbreed	Day 10	40.7 (Day 12)	35%	30%	Post. (0.0008)	No treatment	Survived
256056C	Control	Crossbreed	Day 5	41°C (Day 6)	34.4%	3%	Pos. (0.003)	Treated	Dead at 70 days
256271C	Control	Crossbreed	Day 5	40.9°C (Day 7)	33%	4%	Pos. (0.002)	Treated	Dead at 70 days
259577C	Control	Crossbreed	Day 7	40.9°C (Days 7)	29.7%	5%	Pos. (0.002)	Treated	Dead at 77 days
253027C	Control	Crossbreed	Day 6	41°C (Day 6)	32.4%	4%	Pos. (0.002)	Treated	Dead at 63 days
202133C	Control	Crossbreed	Day 6	41°C (Day 6)	27.3%	5%	Pos. (0.0033)	Treated	Dead at 63 days
258165C	Control	Crossbreed	Day 10	40.5°C (Day 12)	31.3%	5%	Pos. (0.0033)	Treated	Dead at 70 days

Animal ID	Group	Breed	Start of fever (day after tick infestation)	Maximum temperature (°C)	PCV / Hematocrit before Challenge	Lowest PCV observed/ Hematocrit	Maximum parasitemia	Treatment	Fate
256255	Vaccinated	Crossbreed	Day 6	39.9°C (Day 7)	40%	20,5%	Post. (0.0004)	No treatment	Survived
256310	Vaccinated	Crossbreed	Day 6	40°C (Day 10)	34.2%	19%	Post. (0.0004)	No treatment	Survived
10C	Control	Crossbreed	Day 10	41.5°C (Day 14)	34.4%	5%	Pos. (0.002)	Treated	Dead at 49 days
11C	Control	Crossbreed	Day 10	41°C (Days 10)	33%	4%	Pos. (0.002)	Treated	Dead at 49 days
12C	Control	Crossbreed	Day 10	41.5°C (Day 10)	29.7%	9%	Pos. (0.002)	Treated	Dead at 42 days
13C	Control	Crossbreed	Day 10	40°C (Day 10)	32%	7%	Pos. (0.002)	Treated	Dead at 42 days
14C	Control	Crossbreed	Day 10	40.6°C (Day 16)	37.3%	9%	Pos. (0.002)	Treated	Dead at 42 days
15C	Control	Crossbreed	Day 10	40°C (Day 10)	36%	9%	Pos. (0.002)	Treated	Dead at 42 days

Post-mortem examinations revealed that the body cavities of all diseased animals were filled with yellow straw-colored fluid and that petechial hemorrhages were widely distributed on the serous membranes. The lymph nodes, liver and gall bladder were enlarged, edematous and fragile with petechial hemorrhages. Extensive necrosis and hemorrhages were seen in the medulla of the affected lymph nodes. Schizont-infected cells that represent the schizont stages of *T. annulata* were confirmed microscopically in the lymph nodes of animals with acute theileriosis. Multiple necrotic foci, edema and foci of lymphoid cell reaction were reported in the liver sections. The spleens were enlarged, edematous and fragile with petechial hemorrhages. Multiple necrotic foci and depletion of lymphoid elements in both white and red pulps were noticed, associated with hemorrhage and hemosiderosis. Both lungs were emphysematous and dark in color with several areas of hepatization. Also, frothy exudate was found in the bronchi and bronchioles. Microscopic examination revealed alveolar emphysema, lobular interstitial pneumonia, and foci of lymphoid cell reaction. Myocardial section showed multiple necrotic foci associated with hemorrhage and interstitial lymphoid cell reaction (Fig. 3).

Co-detection of other TBPs

RLB assays were performed on blood samples collected from animals before and after vaccination and after challenge. The results revealed that all animals were negative for tick-borne pathogens at the start of the study. Only vaccinated animals turned positive for *T. annulata* after inoculation of the attenuated cell line. After field challenge all animals became positive for *T. annulata*, some of them also tested positive for other tick-borne pathogens. In the vaccinated group, ten animals also tested positive for *Anaplasma marginale* and one animal for *Babesia bovis.* In control group, all animals became positive for *T. annulata*, 16 animals were positive for *A. marginale* and three animals were positive for *Babesia bovis* (Table 3). All co-infections were considered as subclinical because the blood smears and 18S srRNA PCR were negative for all pathogens except for *T. annulata* which confirmed with Tams-1 PCR and sequencing. The sequences were submitted to GenBank and can be retrieved using accession numbers MN223723, MN223724, MN223725 and MN223726.

Table 3
Co-infection with other Tick-Borne Pathogens in both vaccinated and control animals of both breeds:

Pathogens	Vaccinated Animals	Control Animals
T. annulata	24	24
Babesia bovis	1	3
T. annulata + Anaplasma marginale	10	16

Discussion

Tropical theileriosis is a significant obstacle for the development of livestock production in most countries located in tropical and subtropical regions, including Egypt. Ticks and tick-borne disease control in Egypt largely depend on using chemical acaricides, but this procedure becomes limited with development of resistance against acaricides. This situation creates the need for other effective control measures [20,21]. Vaccination against tropical theileriosis using an attenuated cell culture vaccine is an interesting opportunity for control and/or eradication of tropical theileriosis. This approach has been previously reported in several other north African countries, including Sudan and Tunisia, but not in Egypt. [11,18, 22–24]. In this study we described the development of an attenuated cell line of *T. annulata* (Egyptian strain). This Egyptian strain was prepared and attenuated according to previously published protocols [23,24]. The quality of this attenuated cell line and the required dose were also determined based on previous studies. As mentioned, before we found that the attenuation at passage 85 was enough and it was near to the attenuation passage in Sudanese cell line and some Tunisian cell line. On the other hand, it was far from other Tunisian cell lines which need more than two hundred passages to be attenuated and ready to use and this finding confirmed that each locality should have its local strain as described before in many studies [11,18, 22–24]. After vaccination and field challenge, both control and vaccinated animals were infested with ticks. All collected ticks were identified as *Hyalomma excavatum* and *Rhipicephalus annulatus*. This finding was in agreement with previous finding in Egypt which concluded that both previously mentioned species are the most common species in Egypt and especially in the Egyptian oasis [2]. The recorded clinical signs were more severe in the control group compared to the vaccinated group. Control animals gradually became anorexic and died. This finding was in agreement with some previous studies that specified the same clinical signs for *T. annulata* vaccination and challenge trials [1,9,11,25]. PCV results in both vaccinated and control groups did not significantly differ before challenge ($p = 0.4360$) using Generalized linear model (GLM) but in contrast, two to three weeks after the challenge the PCV dropped significantly in the control groups when compared with the vaccinated group ($p < 0.0001$). This result indicates a heavy infection with *T. annulata* in the control group (Fig. 1). This finding is supported by previous finding which indicated severe drop in the PCV in case of *T. annulata* infection [12,22,24,26]. The antibodies' titers raised in all vaccinated animals according to the results obtained by using TaSP-ELISA assay when compared with the data collected from control group. This finding indicated that the attenuated cell line can enhance the production of statistically significant titer of the protective antibodies without significant clinical infection ($P > 0.0001$) [22,24]. The protection rate in the vaccinated groups was 100% and it is closely similar to the finding recorded in China which ranged from 99.5–99.9% [27–33]. Co-infection with other tick-borne pathogens was detected through the RLB assay which is more sensitive when compared to blood smears, PCR, and nested PCR, suggesting that all co-infection occurred at low levels [2,34]. The higher co-infections in nonvaccinated (Control) group when compared with the vaccinated one could be attributed to the heterologous or non-specific immunological effects (NSE) that may be induced when live-attenuated vaccines are used. Several studies concluded that using of an attenuated vaccine can induce protection against other pathogens in both animals and human through the NSE [35–37]. The post-mortem lesions observed in died control animals were similar to the previously mentioned lesions in studies that investigated fatal cases of *T. annulata* infection [11,38]. From the previously mentioned data we could conclude that using of the living attenuated Egyptian cell line infected with *T. annulata* has a potential efficacy in the protection of both exotic and crossbreed cattle against clinical form of *T. annulata* even in highly endemic areas and it should be a recommended vaccine against tropical theileriosis in Egypt as in many other countries to overcome this problem in our Egyptian field. This is also in agreement with previous studies that recommended *T. annulata* attenuated cell line as a vaccine to control tropical theileriosis in many countries [11, 22–24]. However, before starting the logistic steps of wide and field application we also recommend further studies targeting assessment of its polymorphism and the interaction with wild circulating stains. Because cattle which survive an infection with *Theileria annulata* or after immunization with attenuated schizonts of this parasite become effectively immune to challenge with the same parasite strain and are thought to be protected against a heterologous strain of the parasite. In this context, T-cells play a crucial role in both induction and maintenance of the immunity. It has been shown that the generation of cytotoxic T lymphocytes (CTL) is closely related to the control of the infection - macroschizont-infected cells are killed in an MHC class I restricted manner [39,40]. Any strain-specificity induced by immunization is likely to be manifested by CTL [41]. Besides CTLs, CD4 + T-cells also play an important role in protective immunity to *T. annulata* infection. They produce macrophage-activating cytokines such as IFN-gamma which produce mediators such as NO to destroy the intracellular schizonts. The role of parasite-specific antibodies is restricted against extracellular stages of the parasites. Based on the above-mentioned facts, the observed

protection of the animals after immunization with attenuated schizont in our study is also mediated by CTL and CD4 + T-cells. This recommendation is supported with a recent study indicated that there is a great heterogeneity of the field *T. annulata* population in the same geographical area of our study [42,43]. Production of cocktail vaccine including more than one isolate may be required as a further step to overcome the parasite's heterogeneity in the future. Work still ongoing to estimate the duration of immunization and the need for booster doses.

Conclusion

In conclusion, Egyptian *T. annulata* attenuated cell line is efficient and can protect both exotic and cross breed cattle reared in endemic areas against Tropical theileriosis. By using this attenuated cell line as a vaccine, the case fatality rates will decrease dramatically. Although, the obtained results are promising, we still recommend more trials targeting different animals in different areas like Nile Delta and Lower Egypt because these areas may have different epidemiological status which may be affect the target animals, target age or time of vaccination.

Patent

Patent number EG 2019121920A1 'Attenuated Tissue Culture Vaccine Against *Theileria annulata* – Egyptian strain submitted to Patent office, Academy of Scientific research and technology, Ministry of scientific research, Egypt.

Methods

Isolation of T. annulata infected cells:

Heparinized blood and lymph node aspirates were collected from a three-year-old cow naturally infected with *T. annulata.* This cow was admitted to the Teaching Veterinary Hospital, Faculty of Veterinary Medicine, Assiut University from EL-Ghanim district, Assiut governorate. The animal showed typical clinical signs of tropical theileriosis. Her body temperature was 41.5°C, with congested conjunctival mucous membranes and enlargement of the superficial lymph nodes. An aspirate was collected from the prescapular lymph nodes. Following sampling and diagnosis, the cow was treated with Buparvaquone (MSD Animal health, New Jersey, USA) at 2.5 mg/kg body weight deeply intramuscular, followed by a second dose after 72 hours. The animal was also treated with non-steroidal anti-inflammatory drugs (Butafenil, Vetoquinol, France) and antibiotics (Marbocyl 10%, Vetoquinol, France) to control potential secondary bacterial infections.

Preparation and attenuation of T. annulata infected cell line:

Preparation of peripheral blood mononuclear cells (PBMC) from heparinized blood was performed by gradually transferring three mL of blood to a Falcon tube containing an equal amount of Ficoll® 400 (Sigma Aldrich, Germany) followed by centrifugation at 1,800 rpm for 40 min. The PBMC layer was subsequently collected and transferred to a new Falcon tube in which it was washed three times with RPMI 1640 (Cat. No, BE12-115F, Lonza, Switzerland) with a centrifugation step at 2,000 rpm for 10 min between washes. The pellet was subsequently resuspended in 2 mL complete RPMI 1640 media with 10% inactivated fetal calf serum (LSP, UK), 1% Amphotericin B (Lonza, Switzerland), 1% Gentamicin (Lonza, Switzerland), 2% Streptomycin and Penicillin (Lonza, Switzerland). Lymph node aspirates were washed three times in RPMI 1640 and resuspended in 2 mL complete media. Both samples were then transferred to two separate filter-cap tissue culture flasks 25T containing 5 mL complete RPMI 1640 and were incubated with 5% CO_2 at 37°C. The media was changed every 72 hours. *Theileria annulata* schizont-infected leukocytes grew continuously and were passaged under sterile laboratory conditions up to 114 serial passages. The obtained cell line was tested to confirm that it was free from any bacterial, mycoplasma or fungal contamination by the regular inoculation of 100 µL from the cell line on enriched 4% sheep blood agar (BioLab 'BAN20500') and EcoBio, Sabouraud-Dextrose-Agar 4% PH Euro-USP, (BioLab 'ESDA20500') and incubate the plates at 37°C for 48 hours and 25°C for two week, respectively before inspection [44].

DNA was also extracted and tested by the Reverse Line Blot (RLB) assay to confirm that the cell line was free from any other protozoan or rickettsia pathogen [3]. The viability of the cell line was evaluated using Trypan blue (Lonza, Switzerland) exclusion counting before storage of each passage in liquid nitrogen and prior to preparation of the vaccine doses Cell viability ranged between 97–99% before storage and vaccination[22–24].

Experimental animals

Twenty-four exotic breed (Friesian) six-month old male calves were purchased from Assiut's official governmental farm, which is located approx. 50 km north of Assiut city. Twenty-four crossbred male calves of the same age were purchased from a private farm in Arab EL-Awamer village, which is located approx. 75 km north to Assiut city.

Preparation of animals for vaccination trail

All calves were kept in tick-free pens for 6–8 weeks prior to immunization. During this time, they were regularly observed and examined to confirm that they were free from any disease or parasitic infection. Blood samples from each animal were regularly collected and examined before and during the field challenge. Complete blood counts (CBC) and Giemsa-stained blood smears were made to assess the animal's health status. PCR assays targeting the merozoite-piroplasm surface antigen 1 (Tams-1) of *T. annulata* and the 18S rRNA gene of *Babesia* and *Theileria* species were performed to test the animals for the presence of *Babesia* and *Theileria* infections. In addition to this, all animals were screened by an indirect *Theileria annulata* surface protein (TaSp) ELISA to check for previous *T. annulata* exposure prior to the start of the vaccination trial [8,26].

Immunization

Both crossbred and exotic groups were divided randomly into two equally sized subgroups (vaccination and control groups). Each animal in the vaccination group was inoculated subcutaneously with 4ml (1 x 106 cells/mL) of the attenuated cell line at passage 85 in the middle third of the neck. Passage 85 has been shown to be the highly immunogenic passage that stimulates a strong immune response in the vaccinated animals during our pretrials to find the most effective passage that will be able to stimulate the immune system to produce protective antibodies against the parasite without any adverse effects.

Animals of the control group were injected with RPMI 1640 culture media only. Both groups were kept in tick-free pens for 3–4 weeks under continuous clinical, hematological, and parasitological monitoring including both molecular and serological examinations that were performed as described above. Both groups were subsequently transported to EL-Wady EL-Geded governorate, a region considered to be highly endemic for *T. annulata*, where they were kept for three months under natural conditions and exposed to field challenge [1].

Field monitoring

Calves were observed daily to assess the tick infestation rate and to monitor the development of clinical signs. The rectal temperature was recorded three times per day: before sunrise, at noon and after sunset. The mean of the three values was considered as the daily body temperature. EDTA blood was collected three times per week and examined for the presence of *T. annulata* development stages. Lymph node aspirates from animals that showed enlarged lymph nodes were collected and used for the preparation of Giemsa-stained lymph smears, which were subsequently examined microscopically. The parasitemia's percentage in each animal was calculated using the following equation (Parasitemia % = (Number of the parasitized RBCs/12500 RBCs x100 which represent the total number of RBCs in 25:50 Microscopic Fields). Serum samples were collected every month for screening using the TaSp ELISA [9,25]. PCR was used for detection of *T. annulata* infection in the blood samples collected from animals before and after immunization and challenge in both groups [45,46]. Positive PCR products were purified using QIAGEN PCR purification Kit (Cat. No. 28104, Qiagen, UK) according to manufacturer's instructions and sequenced in both directions using an ABI 310 Sequencer at the Molecular Biology Research & Studies Institute of Assiut University [47]. Sequences were subjected to BLAST similarity searches. A RLB assay for the simultaneous detection of *Theileria/Babesia* and *Anaplasma/Ehrlichia* was employed to determine if tick-borne pathogens other than *T. annulata* were present in the blood samples [2,3].

Necropsy finding

Postmortem examinations were performed in the Department of Pathology and Clinical Pathology, Faculty of Veterinary Medicine, Assiut University on calves that died during the experiment [14].

Tick collection and identification

Ticks were manually collected from animals using a forceps and identified morphologically according to standard morphological keys [48].

Statistical analysis

General linear model analysis was conducted in R. Pairwise analyses were attached by least square means analyses for multiple comparisons under the lsmean package with Tukey adjustment. Significance levels were interpreted as: P-Value ≤ 0.00*** (Highly significant),0.001** (Moderate significant),0.01* (Mild significant), 0.05 (Non-significant) [49–51].

Ethical approval

The study is reported in accordance with ARRIVE guidelines. the working protocol, procedures, and all the experiments were ethically reviewed and approved by the Scientific Research Committee and Ethics Board of Assiut University, Egypt, approval number is IRB no: 04-2022-300018. The working protocol, procedures, and all the experiments conformed to recognized standards of Animal research applied by Assiut University and the animal welfare code in Egypt. Additional ethical approval was obtained from both Assiut governorate (Assiut University), 71526, (616, 24.02.2019) Egypt and New Valley Governorate (686, 2.3.2019) and the Veterinary authorities in the New Valley Governorate (485, 3.3.2019).

Declarations

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DFG project “Molecular epidemiology network for promotion and support of delivery of life vaccines against *Theileria parva* and *Theileria annulata* infection in Eastern and Northern Africa” (DFG SE862/2-1 and DFG CL166/4-2).

Author Contributions: AAH, AMR, LS and JA planned and coordinated the study. AAH and AMR performed field and laboratory work. SF carried out the statistical analysis. All authors analyzed the data. AAH drafted the manuscript. All the authors critically revised the manuscript. All authors read and approved the final manuscript.

Additional Information

Conflicts of Interest: The authors declare no conflict of interest.

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Data Availability Statement:

All data generated or analyzed during this study are included in this submitted article.

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Figures

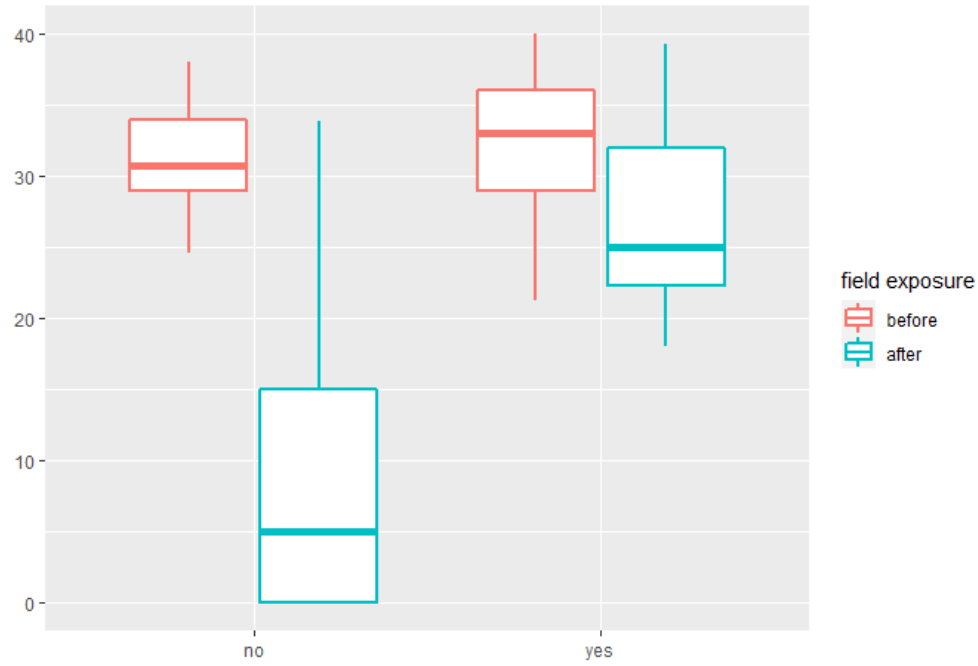

Figure 1

Statistical analysis for the PCV results in both groups before and after challenge. **X-axis represents the mean of PCV values and Y-axis represents the animals' group (No=** represents the mean distribution of PCV for not vaccinated calves **& yes =** represents the mean distribution of PCV for vaccinated calves**)**. P-value before challenge ($p = 0.4360$) and P-value after challenge ($p < 0.0001$)

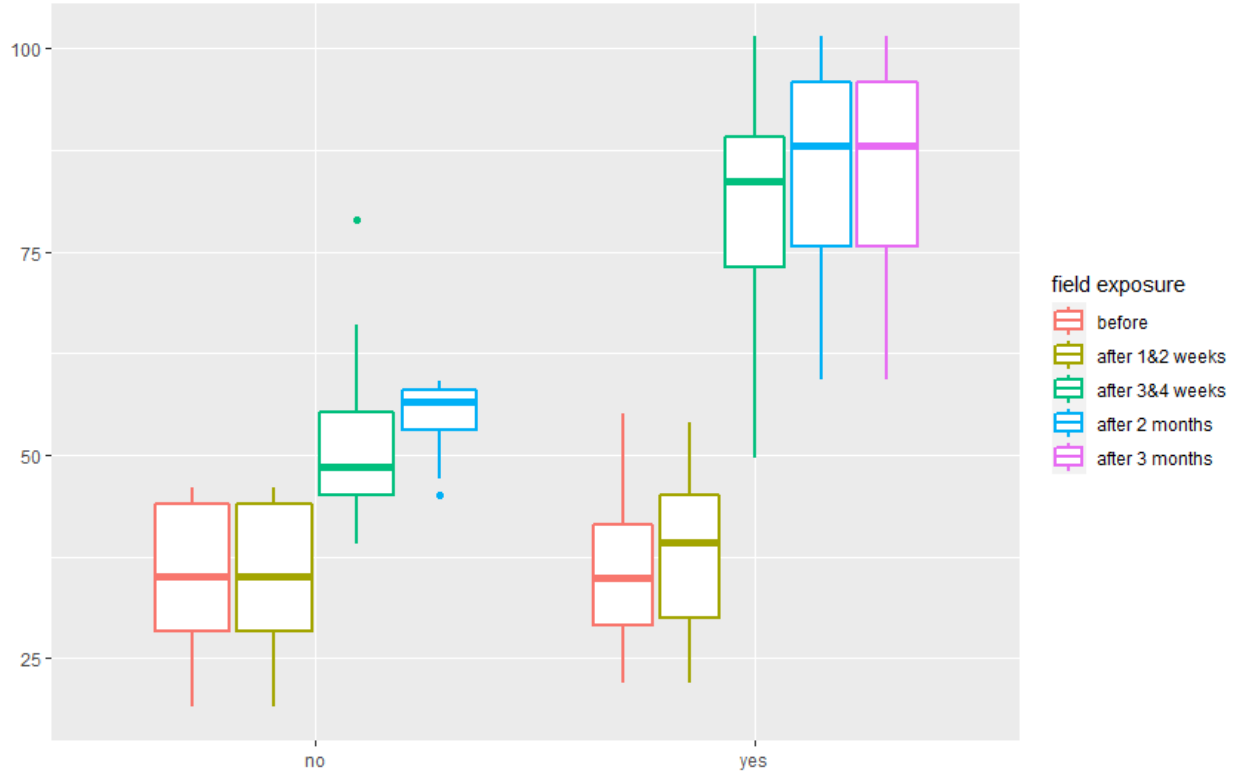

Figure 2

Statistical analysis for the TaSp ELISA results in both groups before and after challenge. **X-axis represents the mean of percent positivity (PP) of TaSP ELISA, and Y-axis represents the animals' group (no=** represents the mean distribution of Abs' PP in not vaccinated calves before and after the challenge **& yes =** represents the mean distribution of Abs' PP in vaccinated calves before and after the challenge**)** (P>0.0001)

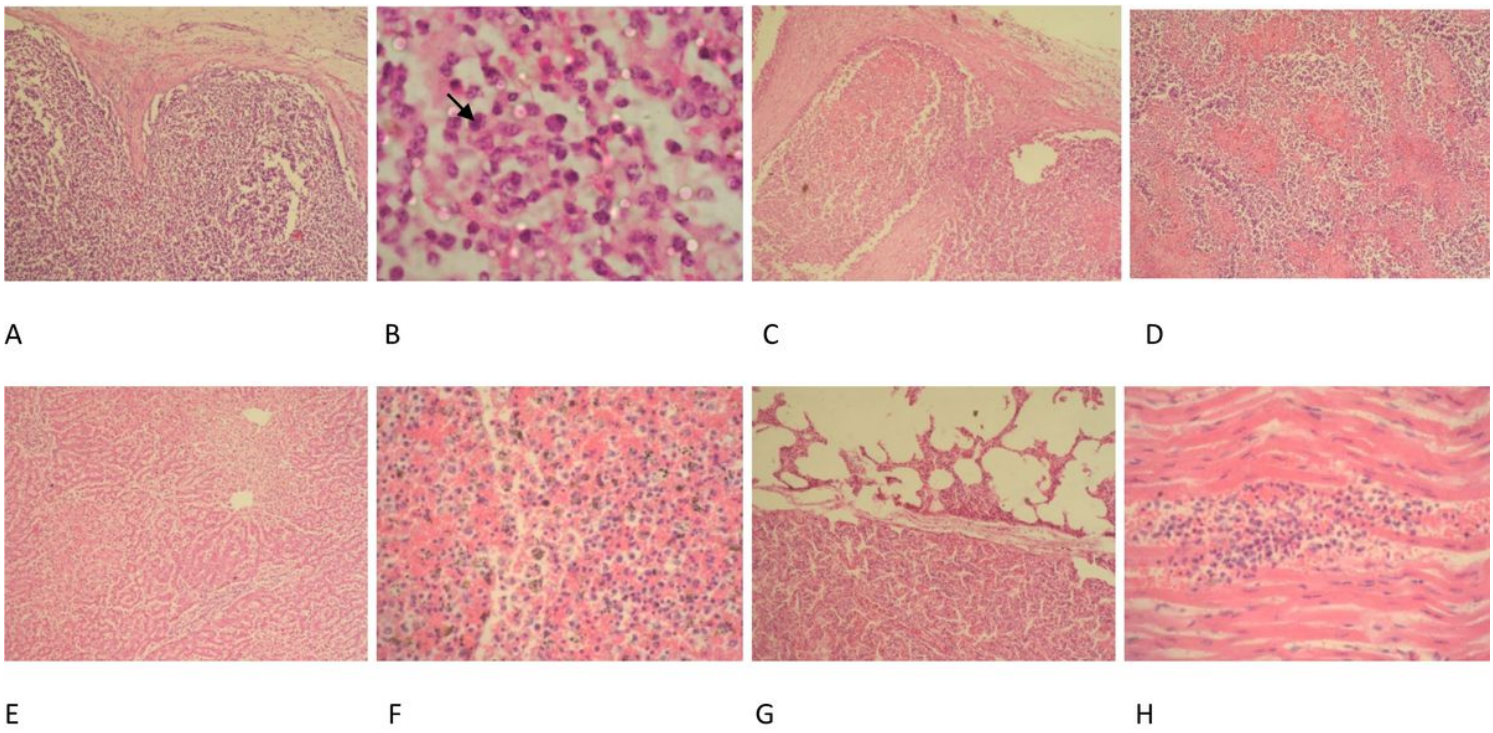

Figure 3

Microscopic finding of the lesions founded in the dead control animals, (A) Lymph node section from cattle with chronic theileriosis showing only depletion of lymphoid elements in the cortex. H&E. X10. (B) Lymph node section from cattle with acute theileriosis showing extensive necrosis and hemorrhage in the medulla with presence of schizont-infected cells (arrows). H&E. X100. (C) Lymph node section from cattle with acute theileriosis showing extensive necrosis and hemorrhage in the cortex. H&E. X10. (D) Lymph node section from cattle with acute theileriosis showing extensive necrosis and hemorrhage in the medulla. H&E.X10 (E) Liver section from cattle with acute theileriosis showing multiple necrotic foci. H&E.X10 (F) Spleen section from cattle with acute theileriosis showing necrotic foci associated with hemorrhage and hemosidrosis. H&E. X40. (G) Lung section from cattle with chronic theileriosis showing alveolar emphysema and lobular interstitial pneumonia. H&E. X10. (H) Myocardial section from cattle with acute theileriosis showing necrotic foci associated with hemorrhage and lymphoid cell reaction. H&E. X40.