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Attenuation of two Field Isolates of *Theileria Annulata* and their Potential as Vaccine Candidates against Tropical Theileriosis in the Sudan

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ABSTRACT

Two *Theileria annulata* schizont infected cell lines (Atbara and Hantoub) isolated from the field in Sudan were serially passaged *in vitro*. Testing of the cell lines at passage levels 90 and 75 for Atbara and Hantoub isolates respectively demonstrated that they were attenuated when inoculated subcutaneously into 10 naïve susceptible calves (five animals for each strain) at a dose rate of $2x10^6$ cells per animal, while five animals were kept as uninoculated control group. Only mild thermal reactions were observed and neither piroplasms nor schizonts were detectable in the inoculated animals. When exposed to natural challenge in an area highly endemic for tropical theileriosis, all animals expressed typical acute tropical theileriosis clinical symptoms. However, only two (33%) of six inoculated animals, exposed to natural challenge, died while all the five (100%) non-inoculated control animals died indicating the potential of the two cell lines as candidate vaccines against tropical theileriosis.

Keywords: Atbara; Cell Line; Hantoub; Sudan; *Theileria Annulata*; Vaccine

I. INTRODUCTION

Tropical theileriosis (*Theileria annulata* infection); is a tick-borne disease of cattle. Tropical theileriosis is an economically important disease of cattle in tropical and subtropical regions (Robinson, 1982) that stretch out from the Mediterranean coastal regions in Southern Europe, Northern Africa including Mauritania, and surroundings of the Nile into Central Sudan and from Middle East to Indian subcontinent, parts of the former USSR and China (Dolan, 1989). *T. annulata* is transmitted by several tick species of the genus *Hyalomma* after cyclical development in these ticks (Uilenberg, 1981) and the distribution of tropical theileriosis parallels that of its *Hyalomma* tick vectors (Dolan, 1989).

The disease in endemic areas in Sudan is highly fatal in exotic breeds and crossbred animals, with little or no mortality in indigenous animals in which clinical symptoms are almost absent except in animals under stress due to pregnancy and/or poor nutrition. *Hyalomma anatolicum* is the main vector of the disease in the country (Salih *et al.*, 2005).

Tropical theileriosis is considered as one of the most economically important cattle diseases in Sudan, causing major losses in livestock production (Latif, 1994; Gamal and El Hussein 2003). It represents a major constraint to the cattle upgrading breeding programs established to foster milk and meat industry in Northern Sudan (Osman, 1992). Thus research towards the control of this disease parasite has long been a top priority of animal health decision makers. The aim of the present study was to assess the degree of attenuation attained by two field isolates of *T. annulata* schizont infected cell lines after *in vitro* serial passage. The study also tested the ability of these cell lines to protect inoculated animals against tropical theileriosis under field challenge in a highly endemic area.

II. METHODS AND MATERIAL

Schizont Cell Lines

Two *Theileria annulata* infected cell lines, namely, Atbara (ATB) and Hantoub (HAN), were isolated from Atbara and Medani towns located in North and Central Sudan respectively (Sharieff *et al.,* 2003). They were serially passaged *in vitro* and their attenuation was assessed by their ability to induce a serological reaction when injected to cattle at low dose (3-4x10^4 cells) and to produce no clinical signs when inoculated into susceptible calves at a dose of2x10^6 cells/animal. At passage 50, Hantoub and Atbara isolates were each inoculated into two susceptible Holstein Friesian calves to test their attenuation. Severe clinical reactions were noticed in the animal inoculated with the Atbara isolate but to a lesser degree with the isolate from Hantoub. It was decided that further passages and testing were required and serial passage of the lines was continued to passage levels 90 and 75 for Atbara and Hantoub cell lines, respectively (Sharieff, unpublished data).

Inoculation of Calves with the Cell Lines

A total of fifteen clinically healthy, naïve, Holstein Friesian calves were used for the safety test and for assessing the immunogenicity of two *T. annulata* infected ATB and HAN cell lines. The animals were randomly divided into three groups of five animals each: two groups were inoculated with ATB and HAN cell lines and the third was kept as a control group. Each calf in the inoculated groups received subcutaneously in the right side of the neck near the prescapular lymph node, a single dose of 2x10^6cells of the designated *T. annulata* infected cell line freshly prepared from cultures as described by Darghouth *et al.* (1996).

Field challenge of Calves

Six animals; three each of the inoculated groups and the five control animals were challenged at day 30 post-inoculation by exposing them to a natural *Hyalomma anatolicum* infestation at the University of Khartoum farm (Shambat Farm, Khartoum, Sudan). This farm had already been proved to be enzootic for tropical theileriosis by introducing three sentinel susceptible calves in one of its premises and two weeks later, 2 out of the three sentinel calves died of typical tropical theileriosis clinical episodes (Sharief, unpublished data). The animals were monitored for development of clinical theileriosis over one month period.

Clinical Parameters

Calves were monitored over a period of 30 days post-vaccination. Rectal temperature values were recorded daily during morning hours and temperature values above 39.5°C were considered as fever. Thermal reactions of calves were further assessed according to the duration of fever into mild (<2-3 days) or severe (>3 days). Prescapular lymph nodes were examined before and after inoculation three times a week for a period of 30 days and its enlargement was quantified as (-) no enlargement, (+) slightly enlarged, (++) moderately enlarged, and (+++) quite enlarged (Beniwal *et al.,* 2000).

Piroplasms in Giemsa stained thin blood smears and Giemsa stained schizonts in lymph node biopsy smears were investigated three times before inoculation. All smears were examined under oil immersion objective lens of a light microscope.

Hematological Parameters

Whole blood samples were collected from the jugular vein in a vacutainer tube containing EDTA. Packed cell volume (PCV) values were determined before cell lines inoculation and once a week for 4 consecutive weeks after inoculation (Schalm *et al.,* 1975).

The cell lines was considered safe if (i) the drop in PVC was lower than 15%, (ii) the duration of fever was lower than 2-3 days, (iii) no alteration in the body condition of the calves, and (iv) the absence of piroplasms in Giemsa stained blood smears (parasitaemia<0.1%) (Gubbels *et al.,* 200b).

Serological Assays

Blood in plain vacutainer tubes were collected and serum was separated before cells inoculation and once a week after inoculation. Tams-1 ELISA was used for detection of antibodies against *T. annulata* (Gubbels *et al.,* 200a).

III. RESULTS AND DISCUSSION

Results

As shown in Figure 1, the thermal reaction of the inoculated animals was within the normal range of animals inoculated with *T. annulata* attenuated cell lines (Darghouth *et al.*, 1996). The body condition of the inoculated animals remained normal throughout the period of observation. Moreover, no gross enlargements of the superficial lymph nodes were observed in the inoculated animals. Regular blood smear and lymph node biopsy smears from these animals revealed neither piroplasms nor schizonts, respectively. Moreover, no decrease in the PCV value was observed over the course of the observation period (Table 1). of the inoculated animals sero converted against *T. annulata* as indicated by positive antibodies reaction in Tams-1 ELISA while all of the control animals remained sero negative.

Four calves, two each from the immunized groups, died due tampany before being transferred to Shambat Farm for challenge. Of the remaining six inoculated calves, two (one from each immunized groups) died of acute tropical theileriosis at days18 to 25 post-challenge (Table 2). All five control animals died of tropical theileriosis within three weeks of exposure to challenge. Hence the protection level afforded by the cell lines was 66.7%.

Table 1: PCV values (%) of calves inoculated with two strains of *Theileria annulata* cell lines pre and post inoculation.

Haematological parameter	Cell line strains			
	Hantoub		Atbara	
	Pre	Post	Pre	Post
PCV mean (%)	24.8	31.2	26.4	28.8

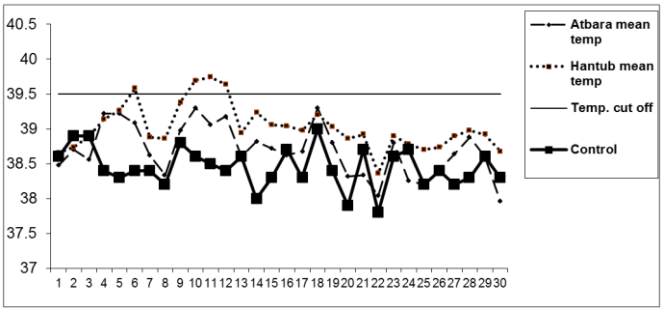

Figure 1 : Daily body temperatures of calves immunized with Atbara and Hantoub isolates

Table 2: Summary of challenge experiment of susceptible calves immunized with $2x10^6$Hantoub (Passage 72) and Atbara (Passage 90) *Theileria annulata* attenuated cell lines

Calves' group	Outcome
Hantoub	
1	Survived
2	Died
3	Survived
Atbara	
1	Survived
2	Died
3	Survived
Control	
1	Died
2	Died
3	Died
4	Died
5	Died

Discussion

Tropical theileriosis causes severe and often fatal disease to exotic cattle breeds, their crosses and susceptible indigenous breeds. An estimated 250 million cattle are at risk of the disease in the endemic areas as estimated in 1990 (Brown, 1990). The disease is a major constraint to livestock production and improvement in the endemic countries (Osman, 1992). Chemical acaricides have been widely used to control ticks and tick-borne diseases around the world, however, development of tick resistance against acaricides, environmental pollution and their rising costs, limited the use of chemical acaricides. Instead, an integrated approach to control ticks and tick-borne diseases (that includes tick resistant animals and anti-tick vaccines and pathogen vaccines) is now envisaged for better control of these conditions.

In the present study, a successful attempt of attenuation of two local *T. annulata* isolates is reported. These isolates attained a reasonable attenuation levels at passages 74 and 90 for HAN and ATB cell lines respectively. The isolates produced no adverse effects when inoculated subcutaneously into susceptible naïve calves at a dose rate of 2 x 10^6 live schizonts infected cells and neither piroplasms nor schizonts were detected

in them using Giemsa stained smears. The cell lines were also immunogenic as indicated by production of specific antibodies in the inoculated animals. In addition, the strains were proved free of bacterial and fungal contamination after inoculation into appropriate media at the department of Biological products of the Central Veterinary Research Laboratory, Soba (Khartoum, Sudan) (data not shown).

The experimental animals were exposed at 30 days post inoculation to challenge under field conditions in a farm known to be endemic for tropical theileriosis during 30 days. However, the level of tick challenge encountered at this farm was unexpectedly extremely high. Literally, the animals were infested by thousands of ticks of all stages. All five control calves introduced along with the inoculated animals died of acute tropical theileriosis within 3 weeks. In the immunized calves; although infected by *T. annulata,* four out of six calves were able to withstand such high challenge and survived. The protection levels afforded by these isolates are certainly underestimated because of the unusual high tick burdens which on one hand might have immune-suppressed these animals and on the other hand, these animals must have also been exposed to high inoculum parasites. Furthermore, in the present investigation we have used a moderate dose to immunize the calves ($2x10^6$ schizont cells) compared to other studies in which higher doses of the vaccines were used ($1x10^7$) (Darghouth *et al.*, 1996; OIE, 2004). A higher dose of our cell lines might have produced a more protective response.

Vaccination against tropical theileriosis using attenuated schizont vaccines was successfully applied in many endemic areas including Israel, Iran, Russia, Turkey, Spain, Morocco, Tunisia, and China [Darghouth *et al.*, 1996; Pipano 1976; Sayin *et al.*, 1997; Viseras *et al.*, 1997; Hashemi-Feshakri 1988; Stepanova and Zablotsky 1989; Ouhelli *et al.*, 1997; Zhang, 1997). The present investigation clearly qualifies Atbara and Hantoub cell lines tested herein as good candidate vaccines. However before embarking on vaccine production and application, thorough studies of the levels of challenge where these prospective candidate vaccines will be used should be undertaken and higher doses should be tested if necessary. Recent studies have indicated great heterogeneity of field *T. annulata* population in the same geographical area of our study (Ali *et al.*, 2006). The production of the vaccine using more than one isolate as we did here is highly advantageous since vaccination failure that may occur could be due to this parasite heterogeneity. Such situation might need administration of a boosting dose of an alternative vaccines to avoid graft *versus* host reaction that are known to occur when using a live *T. annulata* cell culture vaccine (Nichani *et al.*, 1997).

IV. CONCLUSION

In conclusion, the present trial clearly demonstrated the attenuation of two Sudanese *T. annulata* cell lines can be used as candidate vaccines. However more testing is required before they can be approved for this purpose. Also before the application of the vaccine in the field, it is recommended that surveys be carried out to determine the target age group of calves that need to be vaccinated, the appropriate dose for each age group and for different breeds, and to explore the possibility of strategic application of the vaccine if the transmission of the disease is restricted to certain seasons as is the case in other endemic countries.

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VI. REFERENCES

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