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An Evaluation of Hybridoma based Anti-tick Vaccine against H. Dromedarii Tick Infesting Camels

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ABSTRACT

Background: Ticks and tick-borne diseases (TBDs) are considered to be the most serious health problems for animals and humans. This issue prompts veterinarians and researchers to adopt better preventive and control strategies. Therefore, this study aims to develop hybridoma vaccine against *Hylomma dromedarii* infesting *Camelus dromedaries*.

Methods: Myeloma cell culture was administered to the immunized mouse in order to produce hybridomas cells. These cells were then emulsified with Montanide ISA-51 adjuvant. Two doses of the prepared anti-tick vaccine were administered intradermally to camels with an interval of five weeks. The humoral response of experimental animals was measured by indirect enzyme-linked immunosorbent assay (ELISA).

Result: There was a significant ($p<0.05$) decrease in immune responses in tick counts and molting capacity. Overall, 85% efficacy of the developed vaccine was recorded. It is concluded that hybridoma based vaccine is effective in controlling *H. dromedarii* infestation against camels.

Key words: Anti-tick vaccine, ELISA, Hybridoma, Montanide, Ticks.

INTRODUCTION

Hard ticks (Ixodidae family) are obligate ectoparasites that are capable of transmitting deadly pathogens worldwide to wild, domestic animals as well as humans. The direct effects of tick infestation in livestock include blood and weight loss and reduced milk production by the animals. Indirect effects are often associated with ticks as a potential vector source for transmitting various tick-borne diseases (TBDs) of veterinary and public health importance (Dantas-Torres, 2008; Ghoneim, 2020). Babesiosis, theileriosis, anaplasmosis, Lyme borreliosis, viral encephalitis and Crimean Congo haemorrhagic fever (CCHF) are the most common examples of TBDs (Zheng *et al*., 2016).

Most of the tick species belong to two major families of ticks including the Ixodidae (hard ticks) and Argasidae (soft ticks) and only one tick species belongs to the Nuttalliellidae (Barker and Murrell, 2004). *Hyalomma* genus is involved in the transmission of more than 80% of TBDs (Horton *et al.* 2014). *Hyalomma dromedarii* is one of the most prevalent among species of camel, rodent, hedgehog and bird species (Wernery and Kaaden, 2002). Pakistan has one of the most diverse tick faunas in Asia with approximately 53 different species under nine genera (Ramzan *et al.* 2020). Hard ticks have been reported from all provinces of Pakistan including Sindh (Soomro *et al*. 2014), Khyber Pakhtunkhwa (Ali *et al*. 2019), Balochistan (Kamran *et al.* 2021) and Punjab (Sajid *et al*. 2009).

Salivary glands of ticks produce a matrix of cement cone protein antigens (Tiantian Zhang, 2015). The TBDs are transmitted to animals and humans through these proteins (Rizzoli *et al.* 2014). These ticks derived salivary complex proteins enable strong attachment to their hosts and also protect them from host immune system (Hollmann *et al.* 2018). Except for some Ixodes species, many tick species complete the final stage of their life in association with their host and subsequently detach from the host. The ticks consume a large amount of blood from the host over a time span of 7 to 14 days before dropping off (Anderson and Magnarelli, 2008).

Balochistan has a large livestock population. These livestock are the major source of nutrients for a large portion of the population and also serve as the main source of income for local farmer (Jabbar *et al.* 2015). Camel is the second most important animal in the province after sheep and goats. It is an important animal because it can survive under serve hot and dry conditions and several days without availability of water (Schwartz, 1992). About 90% of camels are infested with *H. dromedarii* specie (Apanaskevich and Horak, 2008). This specie has a significant economic impact because it carries Congo virus (Lakhani *et al.* 2002) which causes Crimean Congo haemorrhagic fever (CCHF) among animals and humans in different developed countries like Pakistan.

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Tick infestation in Balochistan is considered to be a major health problem in camels. This issue has been completely ignored by researchers and veterinarians (de la Fuente and Kocan, 2006). Acaricidal use is also limited in this province due to its short shelf life, high cost (de la Fuente and Kocan, 2006; Kamran *et al.* 2021) and chemical residues in meat, milk and the environment. Therefore, ticks are usually removed by bare hands or using forceps. In this way, farmers are also exposed to tick bites and TBDs. For these reasons, an alternative cost-effective approach to control tick infestation has been suggested (de la Fuente and Kocan, 2006) and vaccination or immunological control is regarded as the most promising, environmentally friendly and sustainable strategy (Nuttall *et al.* 2006). Preparation of tick vaccine require selection of an appropriate antigen which minimises the possible tranmiossion of TBDs (Tiantian Zhang, 2015). Production of antibodies can be enhanced using hybridomas cells (Zaroff and Tan, 2019). Hybridoma is the most preferred method and is used to preserves the innate functions of immune cells (Parray *et al*. 2020). The present study is aimed to develop an effective hybridoma based anti-tick vaccine to reduce the tick infestation in camel population of Balochistan.

MATERIALS AND METHODS

Study and sampling design

The current study was conducted in five districts of Balochistan namely Quetta, Sibi, Noshki, Chaghi and Lasbella (Fig 1). These districts were selected because they are located near the border of Afghanistan and Iran and also have geographic importance due to their proximity to the Arabian sea.

Identification of ticks

All stages of collected ticks were examined under a compound microscope (Olympus SZX16 stereoscopic microscope) and their morphological features were identified using available taxonomic keys (Walker *et al*. 2014). The scientific names of identified ticks were validated from the available online databases (http://rafaela.inta.gob.ar/nombresgarrapatas/; http://zoobank.org/). A few samples of adult female ticks were also observed under Scanning Electron Microscope (Hitachi S3400-N, type-II) at 500 µm magnification (Fig 2). Some tick female species were reared under the invitro artificial feeding unit (Ahmed *et al.* 2011).

Extraction of cement cone protein

Cement cones from mouthparts of 14 days fed adult female *H. dromedarii* were carefully dissected using soft tissue forceps. Collected cement cones were rinsed with PBS with 1% of protease inhibitor and then resuspended in 100 µL sterile Tris-buffered saline (TBS, pH 7.2). Samples of cement cone was sonicated, centrifuges at 500 x g for 8 minutes and supernatants were collected. Cement cone proteins extracts were precipitated in methanol and stored at -20°C until analysis.

Hybridoma monoclonal synthesis

Hybridoma vaccine for *H. dromedarii* was prepared by following the standard protocol (Gardner *et al.* 2002). Myeloma cell culture was obtained from the Biochemistry laboratory at Bolan Medical Complex, Quetta. An

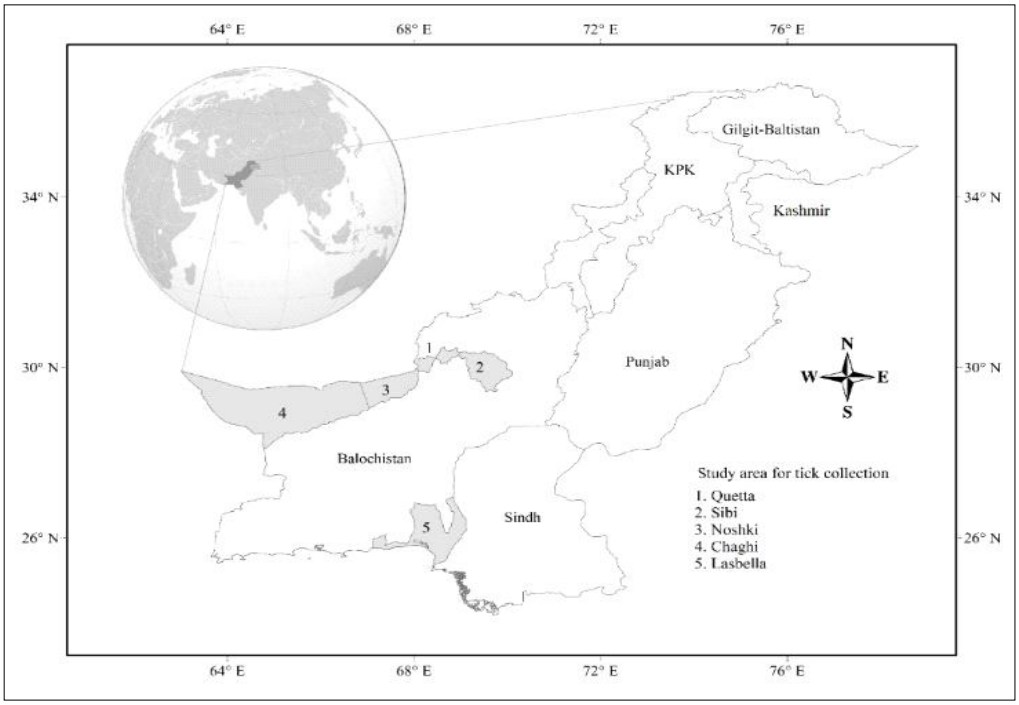

Fig 1: The location of the tick collection areas in the Balochistan province, Pakistan. The map was designed in ArcGIS.

experimental mouse was immunized with salivary cement cone protein. The plasma cells were removed from the spleen of this mouse after three days and fused with immortalized myeloma cells at 37°C for 2 days. Cell fusion resumes in the production of hybridoma. This devopled hybridoma was transferred to the hypoxanthine-aminopterin-thymidine (HAT) medium for 8 days. Hybridoma culture supernatants were screened for antibodies to salivary gland antigens by ELISA. A summary of the developed vaccine is given in Fig 3.

Immunogen preparation

The camels were divided into two groups for evaluation of immunogenic response for hybridomas based anti-tick vaccine. Eight healthy camels were kept in group A (experimental) and five camels were kept in the group B (control). History with tick infestation, TBDs and having positive ELISA reports were adjusted as exclusion criteria for camel selection. Hybridomas mixture was prepared using the emulsification with an equal amount of adjuvant using a homogenizer. First and second doses of vaccine was prepared following the vaccine adjuvant ratio used by Kamran *et al.* (2021). The vaccine was stored at 4°C-8°C for five weeks (WHO, 2015). Blood samples of experimental camels were taken from jugular vein before and after tick infestation. Collected blood samples was centrifuged at 5000 Xg for 10 minutes at 4°C for the separation of plasma and blood cells. Plasma was stored at -20°C till ELISA testing.

Fig 2: *Hyalomma dromedarii*: Electron micrograph of dorsal view.

Sterility of developed vaccine

The sterility of the vaccine was observed using culture media such as Tryptose blood agar, Nutrient agar and Thioglycolate media for 24-48 hours at 37°C. All media plates were reported to be negative for fungal growth as well as for any aerobic and anaerobic bacteria. The red blood cells (RBCs) of the camel were taken in EDTA tubes along with 300 µl/2 ml of crude tick cement cone protein antigens to perform Indirect-Hem Agglutination-Assay (IHA). Diluted phosphate buffer saline (PBS) was added to remove excessive protein and then 1% RBCs were used to determine the antibody titre. Agglutination of the red cell at ≥1:25 was evaluated as positive for antibody presence (Harris *et al.* 2009).

The enzyme-linked immunosorbent assay (ELISA)

The hybridoma mixture was vortexed for 3 minutes and aliquots in 150 µl before being transferred to 96-well polystyrene microtitre plate. Plate was covered with adhesive plastic and incubate at room temperature for 2 hours. The plate was washed twice firstly with 200 µl PBS and then 200 µl of blocking buffer was added per well and incubated at room temperature overnight. About 100 µl diluted primary antibody and 100 µl of the conjugated secondary body (Goat-anti mouse IgG) (Thermo Fisher Scientific, Waltham, MA, USA) were added followed by 50 µl P-para-nitrophenyl phosphate (Merck, Germany). After sufficient colour development within 20 min, the absorbance of end products of the humoral reaction was measured with the light wavelength OD_{405nm} using a microplate reader (BioTek 680,168-1000). At the end of the second vaccination dose,

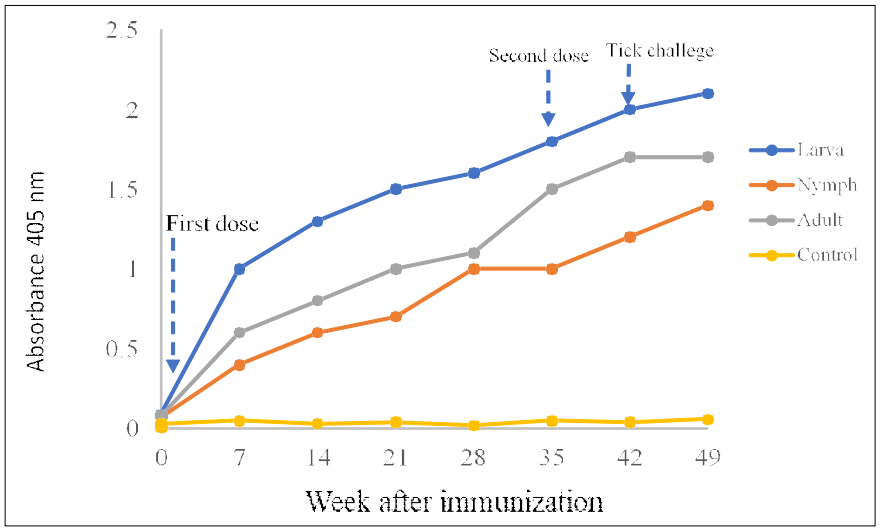

Fig 3: Antibody titers after vaccination doses (*n* =100 instars larvae, nymph and adult). Antibody titration by indirect ELISA; Hybridoma protein antigen was captured with goat-anti mouse IgG and pooled vaccinated camel serum was diluted to calculate endpoint titers. End-point titer cut-off is indicated by circles. Primary and secondary responses are indicated by arrows.

all camels of experimental group were tick challenged with the laboratory strain of *H. dromedarii.*

Statistical analysis

Data presenting negative and positive antibody titres were graphically contrasted in Microsoft Excel, 2019. For results analysis, the biological parameters were examined independently using the molting formula. The values of hematological parameters were expressed as arithmetic mean and standard deviation while comparing to reference levels and data was statistically analyzed using one-way ANOVA. All values were first inserted in Microsoft® Excel-based sheet then data was evaluated using SPSS® 20.0 for ANOVA (Inc., Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Tick prevalence

A total of 273 tick specimens, all from the Ixodidae family were collected from five districts and identified to the species level. The highest prevalence of ticks was reported from Quetta (17.9%) followed by Sibi (16.8%), Noshki (16.1%), Chaghai (23.4%) and Lasbella (25.6%) districts. The ticks were collected from six anatomical regions including the tail (21.6%), belly (20.8%), head (19%), ear pinnae (15%), neck (15.7%) and trunk (7.69%) regions of the camel (Table 1). In our study, tick infestation was highest in the tail region than in other body parts and in agreement with earlier reported results (Abbasi *et al.* 2017; Kemal *et al.* 2016). High tick infestations in certain body parts can be attributed to the fact that ticks prefer warm, moist skin and underneath areas with a good vascular supply (Ogden *et al.* 1998).

Humoral response

The ELISA based antibody titers after five weeks of inoculation showed an increase in antibodies level. This increase in antibody titer was observed to continue for five consecutive weeks. The results demonstrate that the developed vaccine is highly effective in infested camels (Table 3) and may provide protection against every possible pathogen (Hromníková *et al.* 2022). The controlled group had shown a steady increase in tick infestation resulting in persistent diverse humoral or immunological effects on the host body.

Hybridromal anti-tick vaccine efficacy

Approximately 80% efficacy for the vaccine was recorded. One week after vaccination, the engorged ticks started to detach themselves from the host body. A reduced reproduction is reported in our study that indicate the efficacy of developed vaccine against ticks. There is now considerable evidence that an anti-tick vaccine can induce significant immunity in the host body against tick infestation (Willadsen, 2004) and TBDs.

Clinical parameters

A total of eight healthy camels were selected for the immunization trial. An increase in white blood cells was recorded in few experimental camels (Table 2). After vaccination, no serious health complications were reported except for a slight increase in body temperature and limited skin rashes in two experimental camels. The sick camels were given proper medications and after two weeks, a complete recovery was observed. Several articles have reported that tick vaccination is generally safe with rare serious adverse events (Rampa *et al.* 2020; Mansfield *et al.* 2009). It has also been reported that anthropogenic activity has a greater impact on the high prevalence and transmission of TBDs than tick activity alone (Boyer *et al.* 2022). For example, camels are mostly reared in the desert, arid and semi-arid regions of Balochistan province (Iqbal *et al.* 2012). These areas share land borders with neighboring countries including Iran and Afghanistan. Frequent cross-

Table 1: Tick life stage placement on different body parts of the camels.

Life stage	Tail	Belly	Head	Ear pinnae	Neck	Trunk
Larvae	3	2	1	1	0	0
Nymph	4	1	1	0	0	0
Male	3	0	1	0	2	0
Female	49	54	49	40	41	21

Table 2: Hematological response of immunized camels.

Hematological parameters	Min-Max Percentage	Mean±SD Day-1	Mean±SD Day-36	Mean±SD Day-43	p-value
RBC ($\times 10^6$/µl)	7.6-11.0	9.66±0.92	9.43±0.87	9.03±0.80	0.68
MCV	27.5-29.4	27.66±0.15	28.26±0.32	28.93.±0.15	0.00
MCH (g/dl	12.1-13.7	13.03±0.32	12.73±0.41	12.46±0.47	0.30
Total leukocytes count (µl)	2.9-9.7	5.56±0.70	12.36±1.15	13.03±1.15	0.00
Neutrophils ($\times 10^9$/l)	33.0-70.0	54.52±5.08	73.21±3.60	56.06±4.55	0.00
Lymphocytes/$\times 10^3$µl	21.0-62.0	34.13±4.17	35.33±3.91	42.86±2.94	0.04
Basophils/µl	0-3.0	0.00±0.00	0.00±0.00	0.00±0.00	0.00
Eosinophils/µl	0-4.0	0.52±0.27	0.89±0.08	2.76±0.59	0.00
Monocytes/µl	0-7.0	0.65±0.26	1.15±0.28	2.04±0.35	0.01

Table 3: Moulting performance of *H. dromedarii* fed on the un-immunized adult camel.

Level of vaccination trials	Immunized	Control	Percentage (%)**	p-value***
Mean percentage moulted larva-nymph after 3 days	5	31	80.30	0.04
Mean percentage moulted larval-nymph after 6 days	11	92	85.50	0.03
Measurement of moulting of larvae and nymphs**	38	83	48	0.04

*Moulting ability = $\frac{\text{Numbers of consequent period evolving after molting}}{\text{Number of larvae serving up to growth}} \times 100$

**Shows percentage decline in molting from larvae into nymph for experimental ticks fed on immunized camels.

*****Results were considered significant if p>0.05.

border and inter district movements of camels for grazing purposes and their use for small scale transport of goods have increased the potential risk of ticks and TBDs in other domestic animals. Administration of anti-tick vaccines to camels may reduce their susceptibility to TBDs. However, unhygienic environmental conditions and the unregulated movement of camels in any infected area can make them susceptible to TBDs (Alebie *et al.* 2021).

CONCLUSION

Hybridoma vaccine is considered highly protective for camels and inhibit the life cycle of ticks by targeting their larval stage. This vaccine may remain functional for a lifetime against tick attack on camels without any harmful side effects. Primarily younger camels were used in the present study for immunization purposes, because they can show a higher level of immunity against tick infestation. This passive hybridoma vaccination is cost effective and also subsidies harmful effects of acaricides. It is therefore recommended that this effective vaccine may be at commercial scale by the relevant government department to prevent tick infestation in camels in Balochistan.

Conflict of interest: None.

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