Discovery of the ectoparasitic mite *Tropilaelaps koenigerum* Delfinado–Baker and Baker (Acari: Laelapidae) on *Apis dorsata* F., *A. mellifera* L. and *A. cerana* F. in Jammu and Kashmir, India

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Honeybee species *Apis mellifera* L. and *A. cerana* F. have been recorded as new hosts of an ectoparasitic mite, *Tropilaelaps koenigerum* Delfinado-Baker and Baker. The presence of *T. koenigerum* in *A. dorsata* F. colonies has also been recorded for the first time from Jammu and Kashmir, India.

HONEYBEE colonies are attacked by various species of mites which cause considerable damage[1–4]. The primary problem mites are *Varroa jacobsoni* Oudemans, *Tropilaelaps clareae* Delfinado and Baker, *Acarapis woodi* Rennie and *Euvarroa sinhai* Delfinado and Baker. The phoretic mite, *Neocypholaelaps indica* Evans, and the stored product mite, *Tyrophagus longior* (Gervais), are rather harmless. Amongst these, *Tropilaelaps clareae* is a well-known destructive pest of honeybees *A. mellifera, A cerana* and *A dorsata* in most of Asia, while *T. koenigerum* Delfinado–Baker and Baker is a recent discovery in colonies of *A dorsata* from Sri Lanka[5] and *A. dorsata* and *A. laboriosa* from Nepal[6]. This paper reports *A. mellifera* and *A. cerana* as new hosts of *T. koenigerum*. It also records from Jammu and Kashmir, India, for the first time, the presence of *T. koenigerum* in *A. dorsata* colonies.

In subtropical areas of Jammu, India, all the four honeybee species, viz. *A dorsata, A. florea, A mellifera* and *A cerana* co-exist. Fifty colonies of *A. mellifera*, 20 of *Apis cerana*, 14 of *A. florea* and 63 of *A. dorsata* were sampled for the presence of mites from March to October 1993. Examination of debris, bee combs, brood, honey and pollen revealed simultaneous infestations of *Tropilaelaps clareae* and *T. koenigerum* in *A. mellifera* and *A. dorsata*. However, in *A. cerana*, besides these two mite species, *Varroa jacobsoni* was also present. In *A. florea, E sinhai* was present. In addition to the ectoparasitic mites, the stored product mite, *T. longior*, and the phoretic mite, *N. indica*, were also found associated with all the honeybee species. Peak infestations of the mites were observed from March to May coinciding with the peak period of brood rearing activities of the honeybees. In general, infestations of *T koenigerum* in colonies of different honeybee species ranged between 3.0–37.5 per cent with peak infestations during March–May. On an average, the number of *T. koenigerum* in g^{-1} debris of *A mellifera* and *A cerana* colonies was 42.00 ± 4.41 ($n = 20$) and 35.80 ± 3.97 ($n = 20$), respectively. Whereas, g^{-1} comb droppings (obtained after dusting entire or part of the comb on white paper) of *A. dorsata* had 59.60 ± 6.61 ($n = 20$) *T koenigerum*. The spread of this mite to Indian honeybees *Apis cerana, A dorsata* and European bee *A. mellifera* could present a serious problem in Asian bee-keeping.

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Vaccination of hamsters with *Mycobacterium habana* against *Leishmania donovani* infection

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***Mycobacterium habana M. simiae*-serovar-1 a vaccine candidate against leprosy and tuberculosis has been evaluated for its protective efficacy against *Leishmania donovani* in hamsters. Three different preparations of *M. habana* vaccine (live, r-irradiated and heat killed) have provided significant protection against *L. donovani* challenge in hamsters. Of the 3 forms, the heat killed preparation has afforded (82.7%) protection closely followed by r-irradiated (73.2%) and live preparations (57.5%). There has been a considerable degree of *in vivo* cross-reactivity between *M. habana, L. donovani* and BCG antigens in developing delayed hypersensitivity reactions in mice. Possibilities of using *M. habana* preparations for elicitation of functional immunity against visceral leishmaniasis have been disscussed.**

VISCERAL leishmaniasis, a chronic protozoan infection caused by *Leishmania donovani* often attains epidemic dimensions affecting millions across the globe particularly in tropical and subtropical countries. The flagel-

lated protozoan hides inside the macrophages of the victim and strikes at the body's defence mechanism leading to severe impairment of immune systems and eluding almost all therapeutic measures. The unique characteristics of the parasite make it hard to control and therapeutic agents hard to develop.

Time and again vaccines have been the best preventive measure against several diseases and during the past few years concerted efforts have been made to evolve vaccine(s) against leishmaniasis[1,2]. Bacilli Calmette and Guerin (BCG) a non-specific stimulator of the immune system has been widely used with some promise against tumour immunity and other infections[3-9] barring leishmaniasis, where protection has been equivocal[10-13]. Past experiences of BCG vaccination in other diseases have been variable, therefore search for alternative cross-reactive immunogens may be of renewed interest.

Leishmaniasis and leprosy having several common facets in pathogenesis and progression of the diseases, could be cross-linked for devising a common strategy for their control[14,15]. One such immunogenic strain of *Mycobacterium* designated *M. habana* TMC 5135 (*M. simiae* Serovar-1) has been identified at CDRI[16]. A vaccine prepared from the mycobacterium is effective against *M. tuberculosis*, *M leprae* and *M. ulcerans* infections in mice[17-20]. Several proteinaceous antigens are common between *M. habana*, *M. tuberculosis*, *M. leprae* and BCG[21]. These experiences prompted us to investigate the role of *M. habana* and BCG vaccines against *L. donovani* infection in hamsters.

L. donovani strain (Man/IN/Dd8) obtained from PCC Garnham, Imperial College, London was maintained in outbred hamsters (35–45 g). Amastigotes for experimental use were collected from the spleen of hamster infected 6–8 weeks earlier. An inoculum of 1×10^7 amastigotes per 0.1 ml was prepared as described by Pal *et al.*[22]. Promastigotes were cultured in *NNN* (Nicolle, Novy and Mc Neal) tubes using RPMI-1640 as an overlay. The parasites were harvested from 8–10 days old culture (stationary or metacyclic forms) and washed by centrifugation.

In the case of *Leishmania* vaccine, for immunization, the formalin-killed promastigotes were used. The washed promastigotes were suspended in 0.1% formalin for 30 min at room temperature. The treated promastigotes (killed) were washed thrice in PBS to remove formalin and these were counted in a haemocytometer, resuspended in PBS at 5×10^6/0.1 ml concentration and stored at –20°C until use.

In the case of *Leishmania antigen (Leishmanin)*, the washed promastigotes were sonicated in an ice bath for 6 minutes separated by an interval of 2 minutes using sonicator (Soniprep 150). The suspension was left at 4°C overnight for complete extraction of soluble protein. It was centrifuged at 660 g for 30 minutes. The supernatant was aspirated, its protein content was determined by Lowry method[23]. The sonicated soluble antigen so obtained was dispensed into small aliquotes and stored at –20°C until use.

In the case of *M. habana* vaccine, *M. habana* TMC 5135 (*M. simiae* Serovar-1) having three strong markers, the photochromogenicity, strong niacin and catalase positivity, was obtained from Trudeau, Mycobacterial Culture Collection Centre, Sarnac Lake, New York. It was maintained *in vitro* on Lowenstein–Jensen (L–J) medium. Briefly, ten days old culture grown in liquid Sauton's medium in the logarithmic growth phase was washed three times with saline at 4°C and finally suspended at a concentration of 15 mg/ml. A part of the suspension was inactivated to sterility with gamma irradiation from 60 cobalt source at 300 K rads and the other part was inactivated to sterility by heating/boiling at 100°C for 30 minutes. Sterility of the irradiated/killed vaccine was confirmed by absence of any growth upon inoculation on L–J medium, nutrient broth and nutrient agar slants. The r-irradiated and heat killed preparations were stored in suitable aliquotes at –20°C.

In the case of BCG vaccine, BCG (TMC 403) was maintained in L–J medium. Ten days old culture growth was harvested, weighed and diluted at a dose of 1×10^8 AFB/ = 1 mg wet weight. An inoculum of 0.1 ml of the above dilution was injected intradermally to each hamster.

Habanin and Bcgin were prepared in the same way as described for leishmanin.

The immunization protocol involved hamsters in five groups, each having 10 animals. These were taken for vaccination. Three groups (I–III) were vaccinated intradermally with 1.5 mg/animal (1.5 mg weight = 6.27×10^8 AFB = 63.3 μg protein) of r-irradiated, live and heat killed *M. habana* preparations separately. Animals of Group IV were vaccinated with BCG (1.0 mg/animal = 1×10^8 AFB) and those of Group V were kept as unvaccinated control. All the five groups were given challenge on day 21 post vaccination with 1×10^7 amastigotes of *L. donovani* intracardially. The animals were kept under constant supervision and observed for mortality and/or any other changes. 25–30 days later, the animals were biopsied for assessment of splenic parasites load. The experiment was terminated after 50 days of challenge.

For studying delayed type hypersensitivity response, sixty young healthy mice were equally divided into 4 groups. Group I, II and III were injected intradermally with formalin killed promastigotes (5×10^6/animals), BCG (1.0 mg/animal) and r-irradiated *M habana* (1.5 mg/animal) preparations separately. Animals of group IV were sensitized with diluent only which served as control. For elicitation of DTH, every group of mice was further subdivided into 3 sub-groups of 5 animals each. Mice of each sub-group were challenged subcu-

Table 1. Effect of vaccination with different preparations of *Mycobacterium habana* against *Leishmania donovani* in hamsters

Groups sensitized	Dose (mg/animal)	No of animals survived/vaccinated on day 25–30 post-challenge	Parasite burden per 100 cell nuclei (Mean ± SD)	% Inhibition (Mean ± SD)	No of vaccinated animals survived on day 50 of challenge
M habana					
Live	1 5	20/24	17 4 ± 7 79	57 5 ± 18 30	20
r-Irradiated	1 5	26/29	11 0 ± 6 29	73 2 ± 14 9*	26
Heat killed	1 5	9/10	7 1 ± 3 79	82 7 ± 8 39**	9
BCG	1 0	19/29	11 5 ± 8 35	72.0 ± 19 6*	16
Control (Unsensitized)	–	27/28	41 2 ± 23.9	–	7

*Significant.
**Highly significant ($P < 0\ 01$)

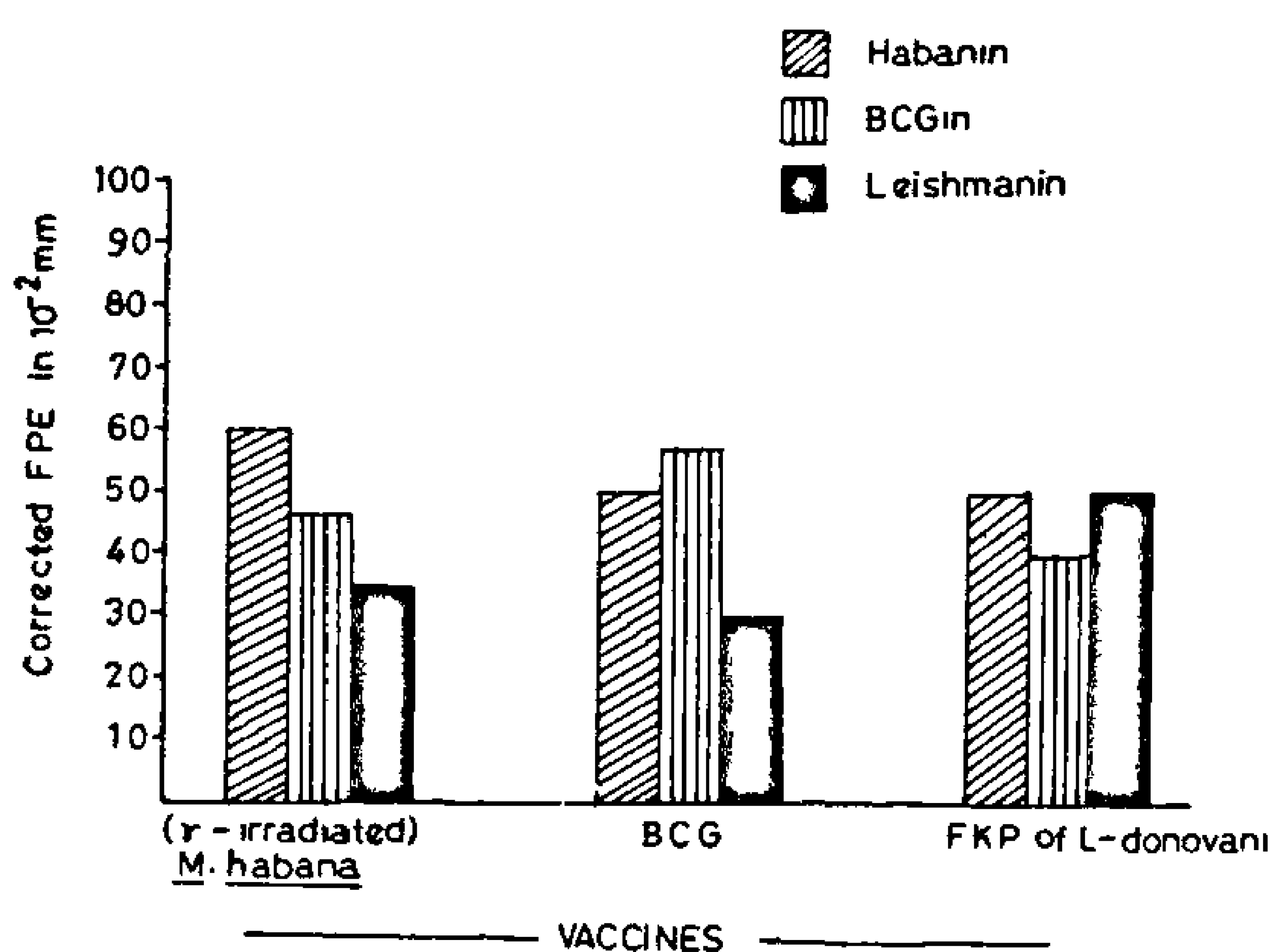

Figure 1. Generation of foot pad enlargement after vaccination and challenge.

taneously in the right hind feet with either habanin or Bcgin or leishmanin (100 µg each/animal). Foot pad enlargement (FPE) was observed at 24 and 48 h of the challenge with sensitins according to Shepard's method[24].

The results presented in Table 1 showed that all physical forms of *M. habana* (live, r-irradiated and heat killed) gave significant protection against *L. donovani* challenge. Of the three preparations, at a dose level of 1.5 mg/animal, the best resistance to the infectious challenge of *L. donovani* was afforded by heat-killed *M. habana* (82.7 ± 8.39%) followed by r-irradiated (73.2 ± 14.9%) and live preparations (57.5 ± 18.3%). BCG (1.0 mg/animal) also provided almost similar protection (72.0 ± 19.6%).

All the groups sensitized either with BCG, *M. habana* (r-irradiated) or *L. donovani* (FKP) elicited positive DTH response when challenged with homologous or heterologous antigens in a criss-cross fashion (Figure 1). There was negligible foot pad enlargement in diluent vaccinated animals against all the 3 eliciting antigens.

The therapeutic measures presently available to combat visceral leishmaniasis are grossly inadequate[25]. The other control strategies – prophylaxis and immunotherapy are yet to emerge. Since vaccines have advantages over therapeutic regimens[26], we ventured to search for effective immunogens against visceral leishmaniasis. *Mycobacterium* spp. are known to potentiate non-specific immune responses of the host and also share antigens common to leishmanial parasites[27]. Since immunity engendered by leishmanial parasites is of considerably lower profile and the parasite cultivation for mass antigen production a tedious task, it will be pertinent that mycobacterium organisms could advantageously be exploited for vaccination against visceral leishmaniasis.

The vaccine made from *M. bovis* (BCG) elicits protection against several infectious diseases including leishmaniasis[13, 28–32]. BCG in combination with leishmania promastigotes has been successfully used to vaccinate school children against visceral leishmaniasis[14]. Despite these commendable findings at times, the action of BCG is not consistent and largely remains unpredictable[27, 33, 34]. The other species of mycobacterium, *M. leprae* has so far defied all attempts to grow in artificial media, thus limiting its commercial viability. *M. tuberculosis* is highly virulent.

M. habana (*M. simiae*-Serovar-1), an atypical mycobacterium has a broad spectrum of action against mycobacterial infections like leprosy and tuberculosis[17–20]. It is non-pathogenic, easily cultivable and possesses heat and irradiation stable protective antigens like *M. leprae*. Thus, this strain was studied for its protective efficacy against visceral leishmaniasis in hamsters. The cross protectivity and cross-reactivity given by *M. habana* against *L. donovani* is presumably

based on the presence of common antigens between the two organisms and this has been experimentally established by SDS-PAGE analysis (unpublished) of antigens of both the micro-organisms. Wherever BCG has been used, it is always in the live stage and killed BCG has no protective value.

The degree of excellence *M. habana* possesses over BCG and the present findings indicate that this strain could advantageously be exploited for elicitation of functional immunity against the fatal disease – visceral leishmaniasis.

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