NRCPD, Japan
(Parent Laboratory)

NRCE, India
(Candidate Laboratory)

Twinning Project on Equine Piroplasmosis – Feedback from Candidate Laboratory

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Equine Population

- **Total world equine population = 58 millions**

	Country	millions
1	USA	9.5
2	China	7.4
3	Mexico	6.2
4	Brazil	5.7
5	Argentina	3.6
6	Mongol	2.0
7	Russian federation	1.3

Impact on Economy

- Horse industry – a report from AHC
  - Direct impact of $39 billion on the US economy
  - Overall impact of $102 billion
  - Additionally, this industry supports 1.4 million equivalent full-time jobs.

Indian Equine Population

Total Indian equine population : 1.77 million

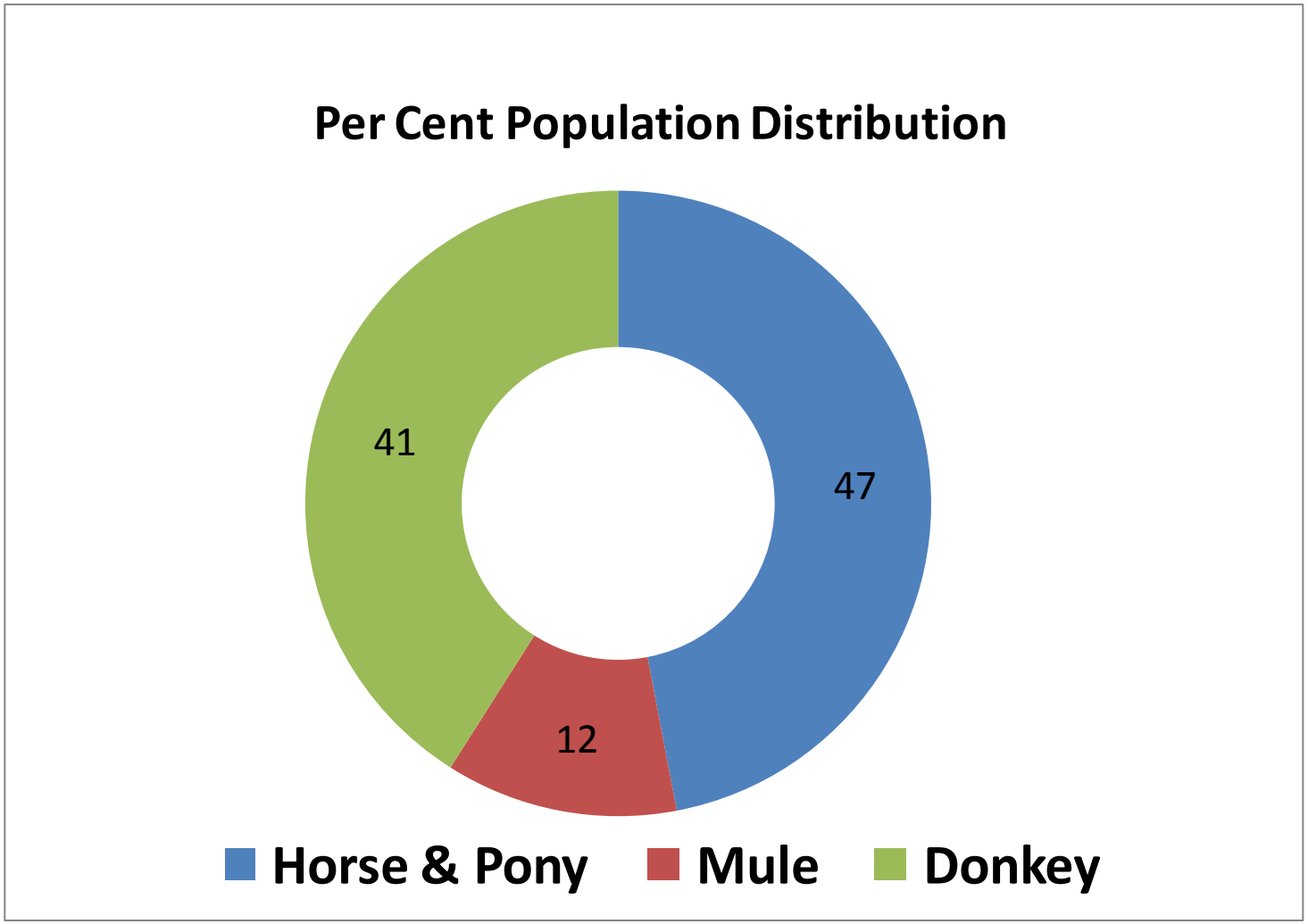
Thoroughbred Horses (~25000 heads)

- **Race-horse industry (~15000 heads)**
- **Other Agencies (~10000 heads)**

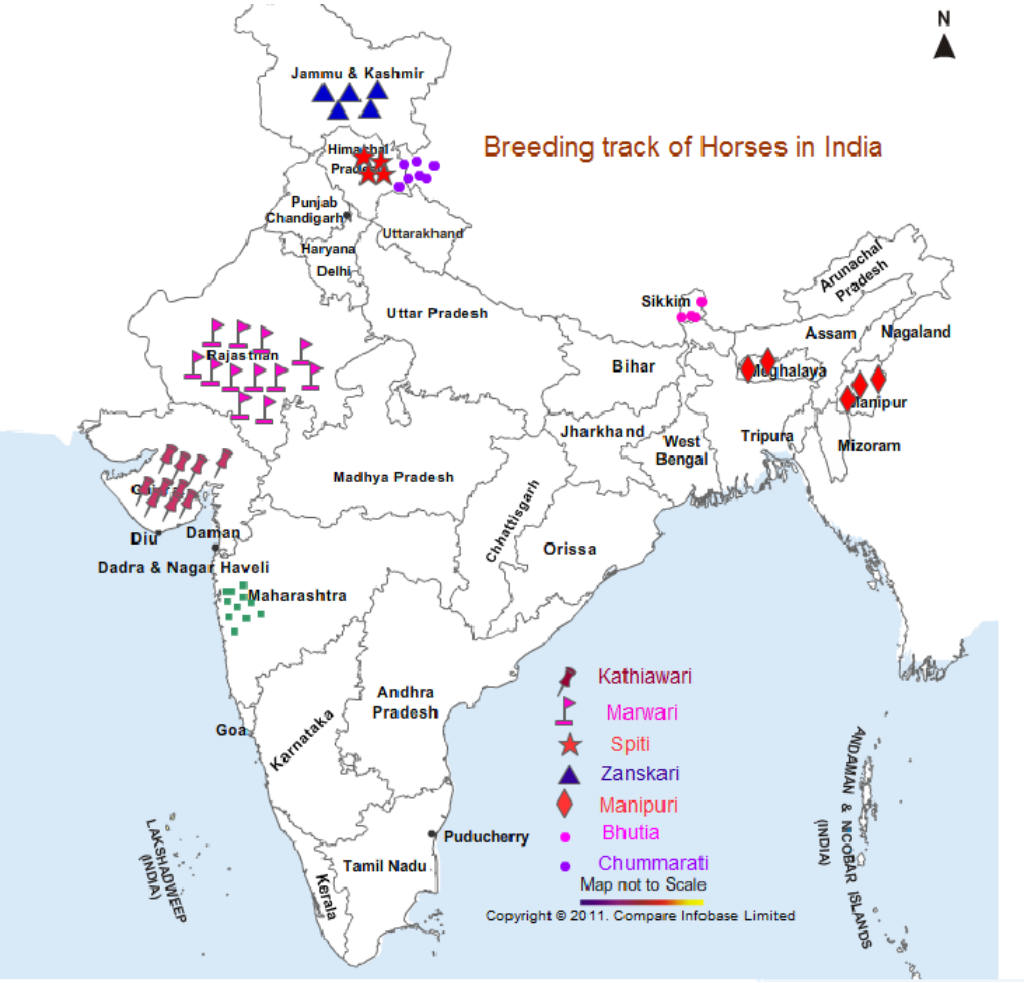
Indigenous Equids (~ 1.735 millions)

- **Defined Indian horse Breeds (~10000 heads)**
- **Non-discript horses, ponies, donkeys and Mules (~1.735 millions heads)**

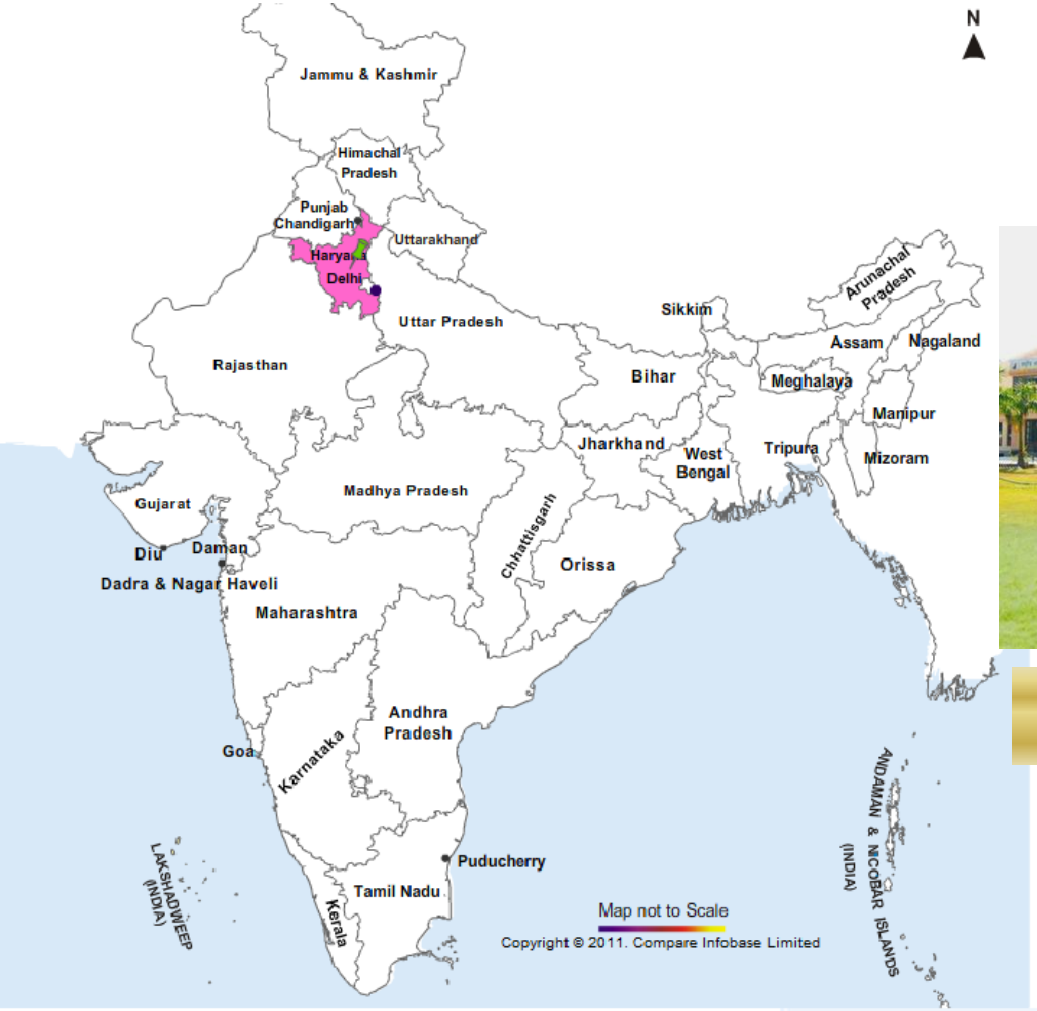
Equine Demography (India)



Breeding Track of Horses in India



National Research Centre on Equine, Hisar



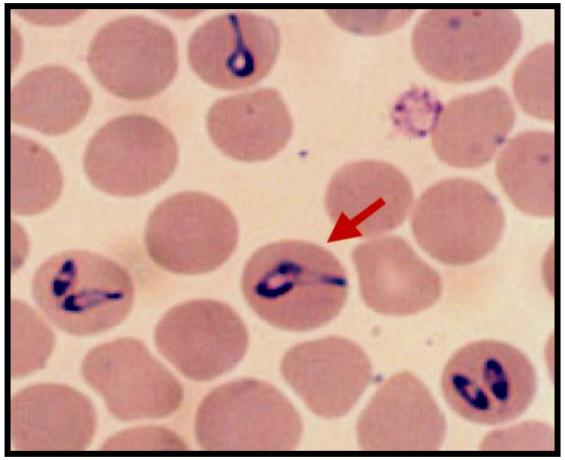
Established: January 7, 1986

Mandate

- To undertake research on health & production management in equines
- To develop diagnostics/biologicals for major equine diseases
- To act as national referral facilities for diagnosis, surveillance & monitoring of equine diseases, and
- To provide diagnostic, advisory & consultancy services

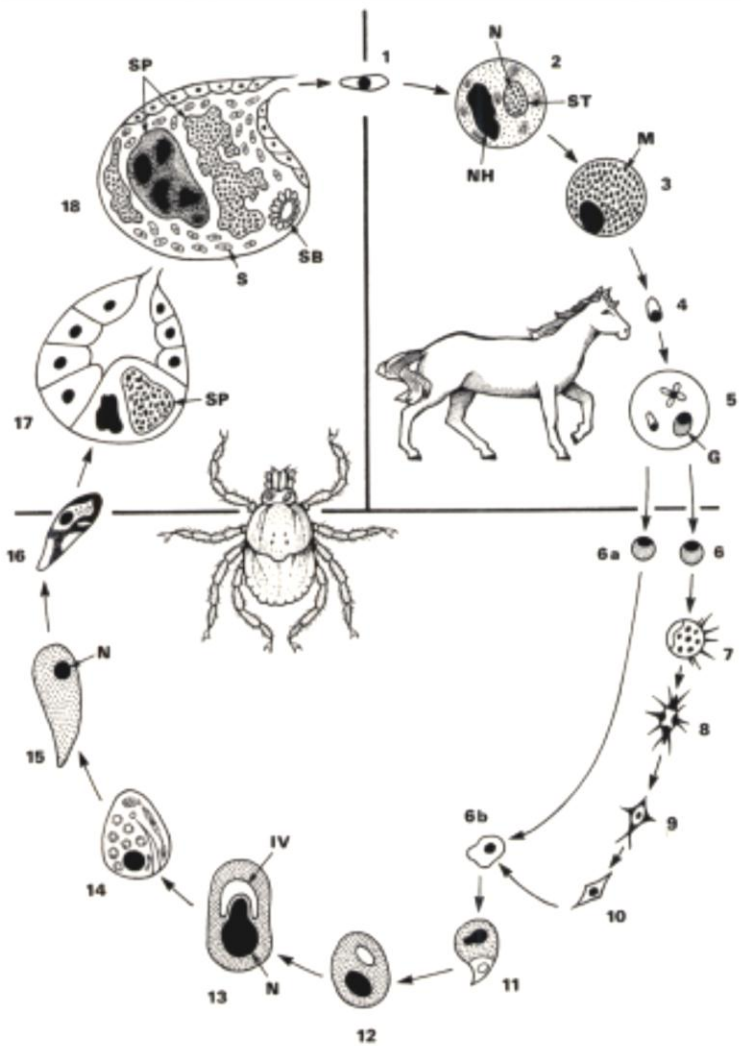
Equine Piroplasmosis

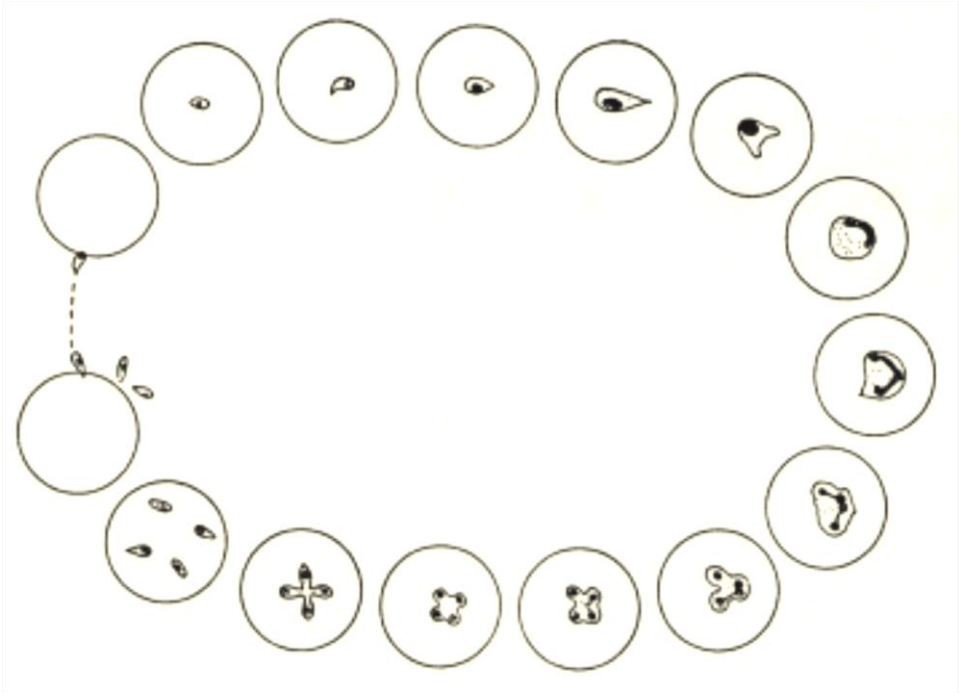
- **Two species**
  - ***Theileria equi***
  - ***Babesia caballi***



- **Prevalent in 90% of the world inhabited by horses.**
- **Only Canada, United States, Australia, Japan, England and Ireland are not considered to be endemic areas**

Life cycle of *Theileria equi*





Different forms of *T. equi* merozoite inside the erythrocytes

Clinical Form

- Acute clinical cases
- Sub-acute
- **Chronic** – non specific clinical signs, mild inappetence, poor performance, drop in body mass, splenomegaly.

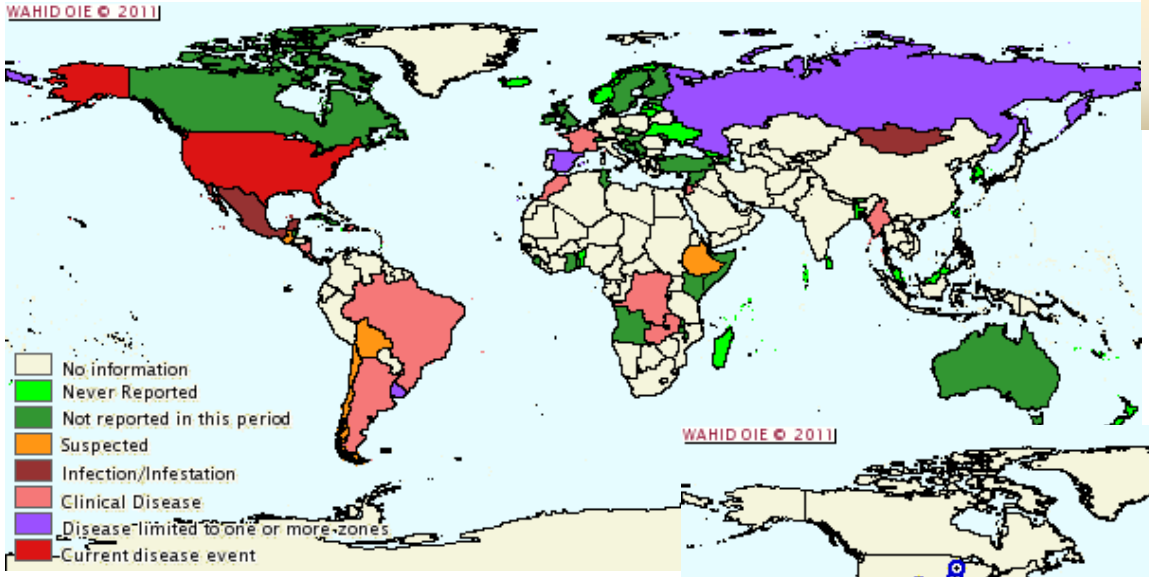
Aborted foetus

Aborted foetus - severe hepatomegaly and splenomegaly

Impact of Latent Infection

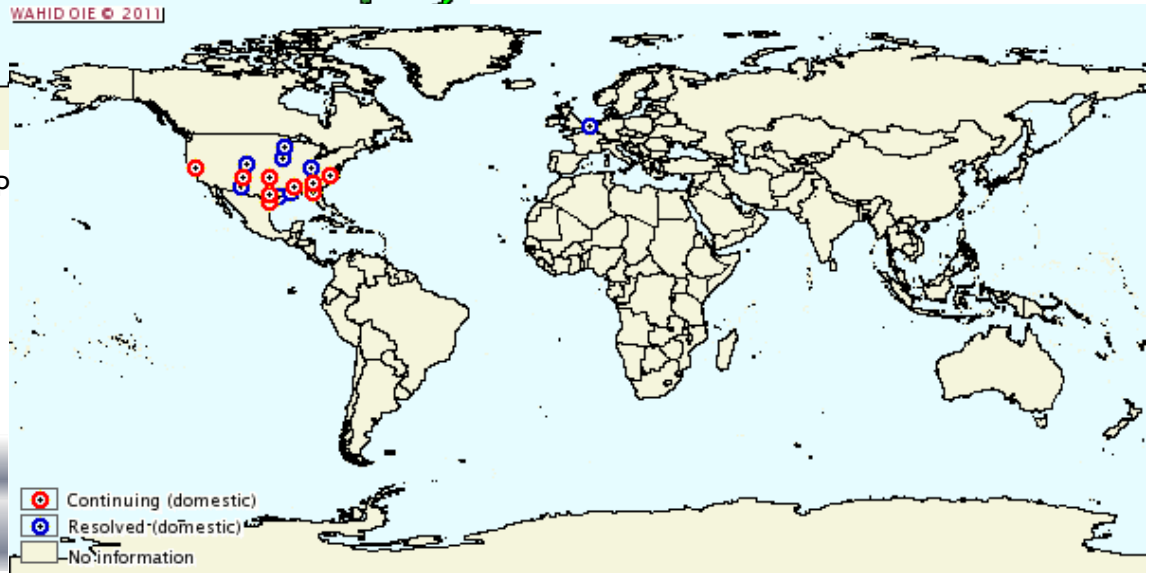
- The latent infection is common in non-descript equids in India.
- These animals act as nucleus herd for maintaining and spread of infection thru vector ticks
  - Diagnosis of sub-clinical infection is of more relevance as these animals remain carrier to the *T. equi* parasite throughout their life span.
- These latently infected animals may exhibit
  - low performance
  - in the event of physical/immunological/mental stress the underline parasitaemia can flare-up leading to clinical form of the disease condition.

World Epidemiology



Disease Distribution Map Jan 2010

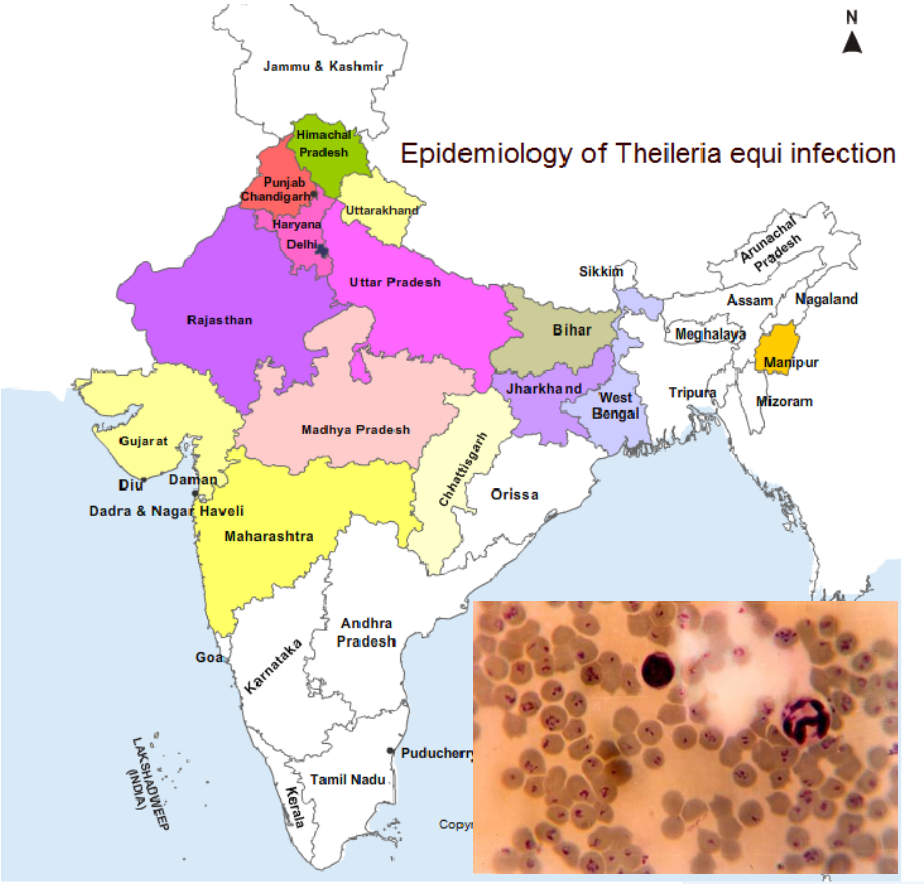
http://web.oie.int/wahis/public.php?page=disease_status_map



Disease Outbreak Jan 2010 to Dec. 2011

http://web.oie.int/wahis/public.php?page=disease_outbreak_map

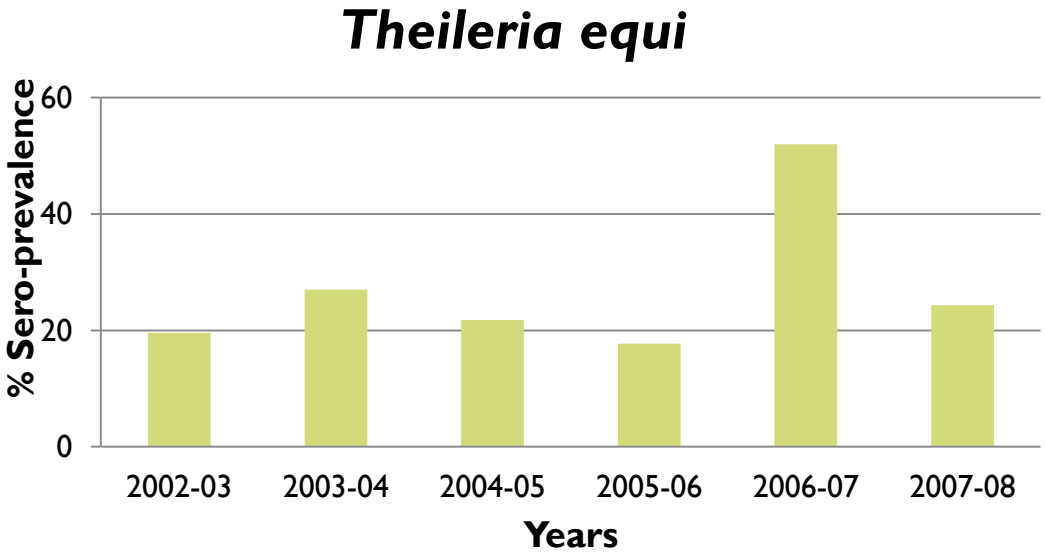
Indian Scenario



- 1976 the Equine piroplasmosis was reported in a outbreak form in imported horses.
- **50.1%** and **49.76%** incidence was recorded in North-West India by CAT & Dot-ELISA.
- Clinical incidence is common in foreign breeds of horses kept in enzootic zones.

National Sero-surveillance Analysis

Year	*Theileria equi*
2002-03	19.6 %, (125 /636)
2003-04	27.04% (387/1431)
2004-05	21.80% (230/ 1055)
2005-06	17.7% (168/955)
2006-07	52.0% (610/1172)
2007-08	24.3% (170/698)
Total	**28.58% (1690/5947)**



Diagnostics

- **Developed at NRCE**
  - Capillary Tube Agglutination Test
  - Dot-ELISA
  - Complement fixation test
  - CCIP
  - Single Dilution ELISA
  - ELISA based on r-Ag.
  - PCR based on EMA-1 & EMA-2
  - MASP in-vitro culture ??
  - IFAT ??
- **OIE approved**
  - IFAT
  - C-ELISA
  - CFT
  - PCR

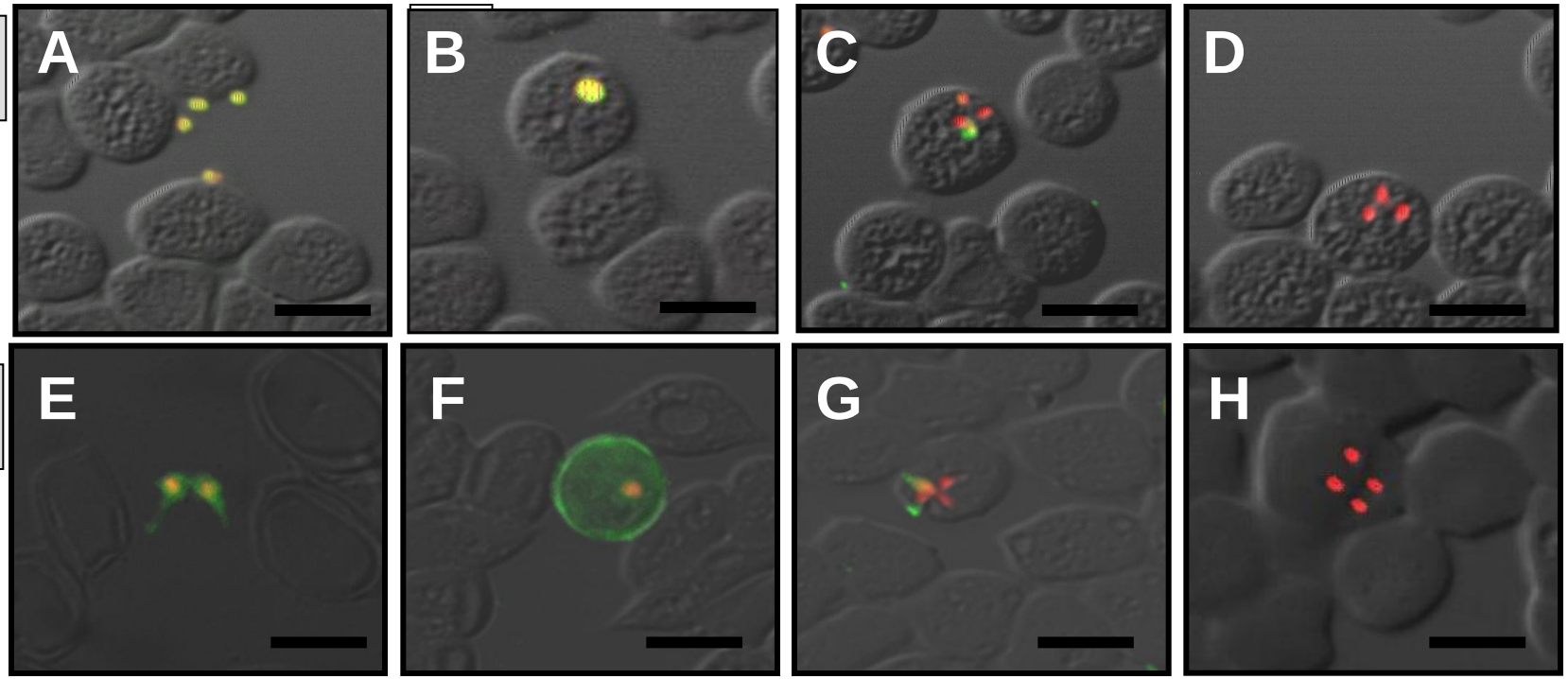
NRCPD, Japan Previous Collaboration

- Under JSPS I joined as Post-doctorate (Nov. 2001 to March 2004).
- We worked on host-parasite relationship of *T. equi* and explored the drug targets for chemotherapy.
  - Kumar S, et al 2004. Expression of Babesia equi EMA-1 and EMA-2 during merozoite developmental stages in erythrocyte and their interaction with erythrocytic membrane skeleton. Mol Biochem Parasitol. 2004 Feb;133(2):221-7.
  - Bork S, et al. 2004. Growth-inhibitory effect of heparin on Babesia parasites. Antimicrob Agents Chemother. 48(1):236-41.
  - Nagai A, et al. 2003. Growth-inhibitory effects of artesunate, pyrimethamine, and pamaquine against Babesia equi and Babesia caballi in in vitro cultures. Antimicrob Agents Chemother. 2003 Feb;47(2):800-3.
  - Bork S, et al. 2003. Growth inhibitory effect of triclosan on equine and bovine Babesia parasites. Am J Trop Med Hyg. 68(3):334-40.

Expression of EMA-1 and EMA-2

Anti-EMA-1 Ab (A to D)

Anti-EMA-2 Ab (E to H)



- during the different developmental stages of merozoite
- detected by IFAT (confocal microscopy)

Twinning Project Objectives

- Capacity building of National Research Centre Equines, Hisar, India so that it can act as a referral centre in the Indian sub-continent with particular reference to SAARC countries.
- Development of sensitive & specific molecular diagnostics (based on recombinant antigens, qPCR techniques) as per OIE guidelines and training of scientific staff from selected laboratories in Indian sub-continent.
- Organization of training programmes/workshops for researchers working in this area of SAARC countries.
- Application submission to the OIE for designating NRCE, India laboratory as OIE reference laboratory for equine piroplasmosis.

Activities under Twinning Project

- **In-vitro cultivation by MASP technique**.
- **qPCR for quantification of parasitic load** in the latently infected equines specially donkeys and horses.
- **Genomic variability** of EMA-1 and EMA-2 gene in Indian equine population, if any.
- **Development of ELISA using expressed protein** from EMA-1 and EMA-2 and its validation on equine samples collected/obtained from different geographical origins.
- Development and validation of **ICT as a pen side field diagnostic test**.
- **IFAT facility** at NRCE, Hisar, India .

In-vitro Cultivation

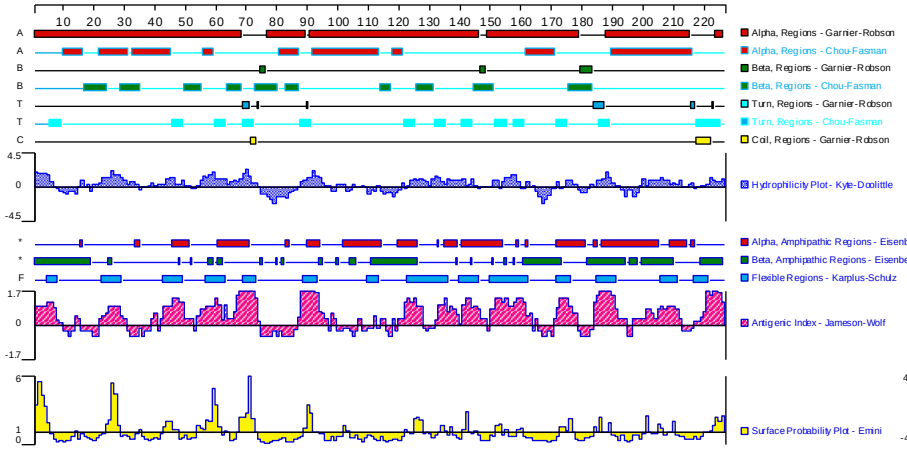
Normal double gas CO_2 Incubator

- Initiated culture, parasitaemia of 7-8% and 5-6% was achieved in *T. equi* and *B. caballi* parasites.

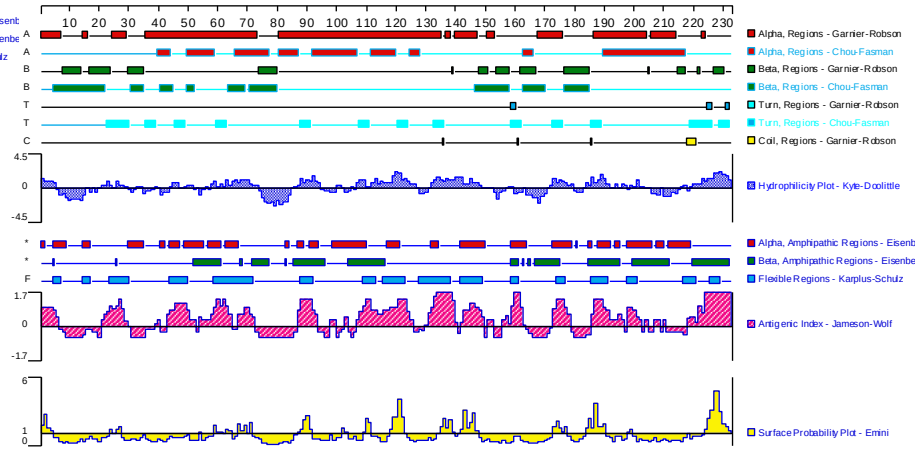
Using Anaero Pouch®

- The parasite were observed on 14th day of the culture [both *T equi* (1-1.5% and *B caballi* (0.5-1%)].
- Upon subculture parasitaemia of 3-4% was observed in both the culture.

Protein Expression

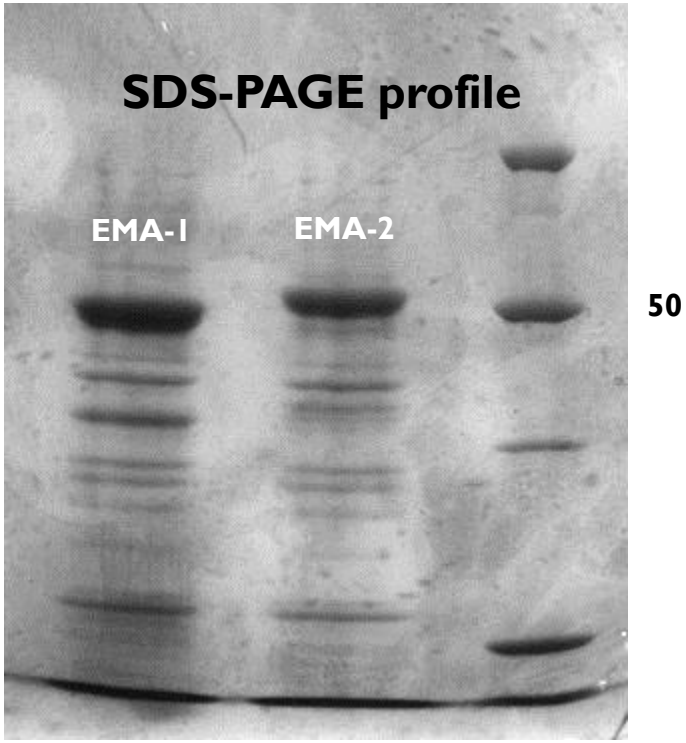


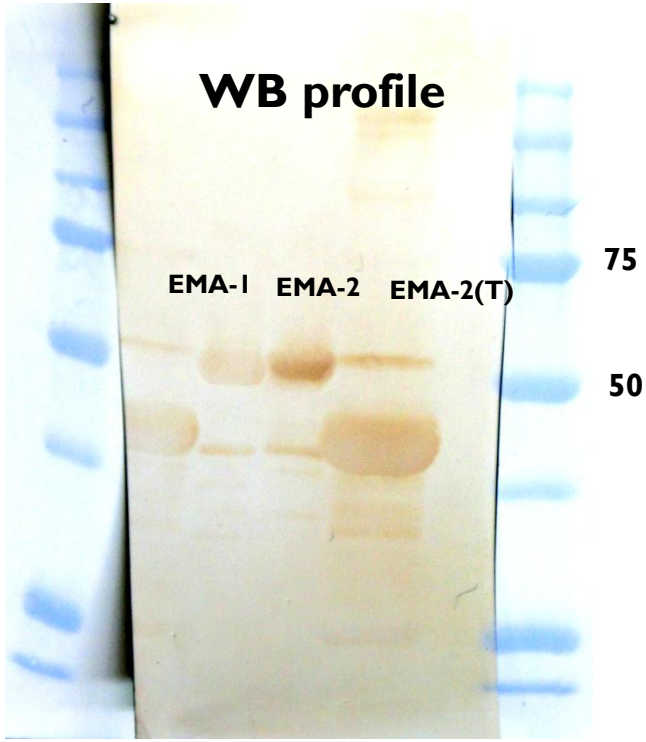
***Theileria equi* EMA-1**



***Theileria equi* EMA-2**

Expression and Immunoblotting





Expressed in pGEX 4T-1 vector **Immnoblotting with +ve serum**

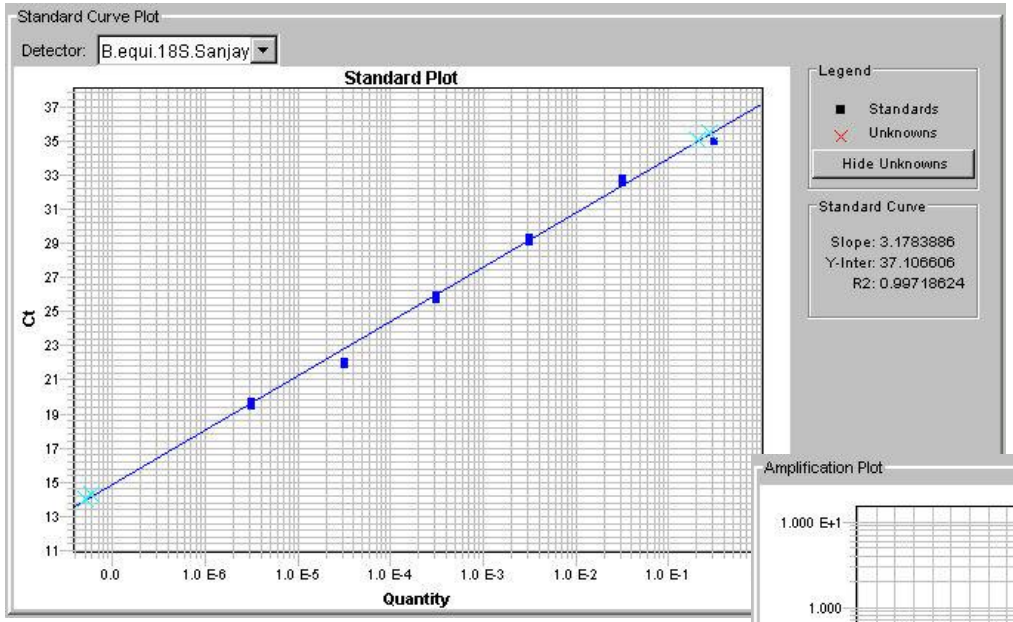
qPCR

Primers for Real Time PCR – 18s RNA (Kim et al 2008)

- Be18SF :5′-GCG GTG TTT CGG TGA TTC ATA-3′
- Be18SR :5′-TGA TAG GTC AGA AAC TTG AAT GAT ACA TC-3′
- TaqMan probe :5′ AAA TTA GCG AAT CGC ATG GCT T-3′

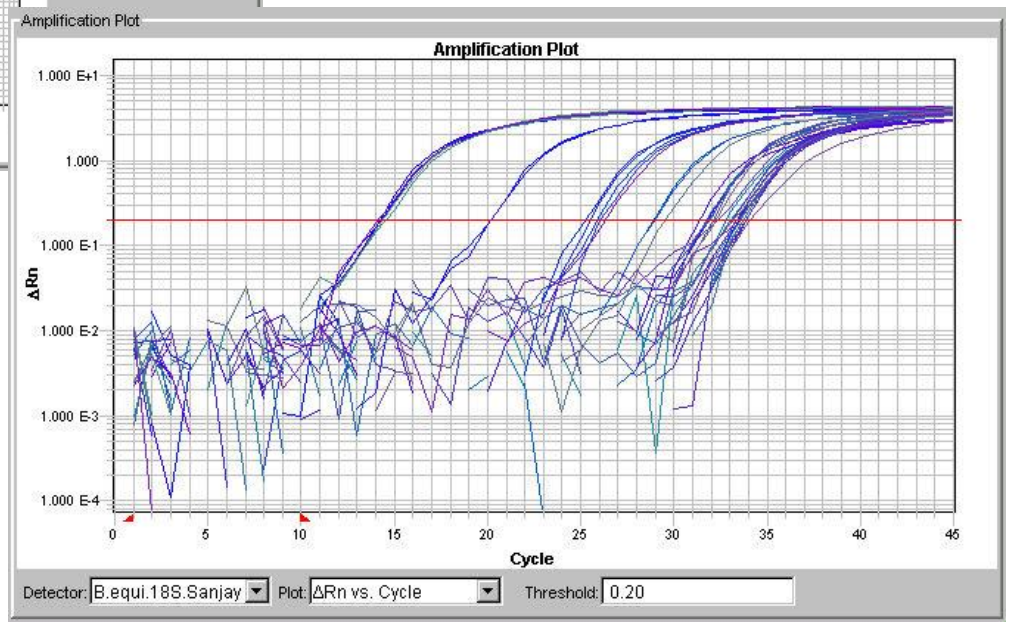
- **More data to be generated using some other primers and probe so as to be used as a diagnostic assay on field samples**

qPCR



Plasmid Copy no.

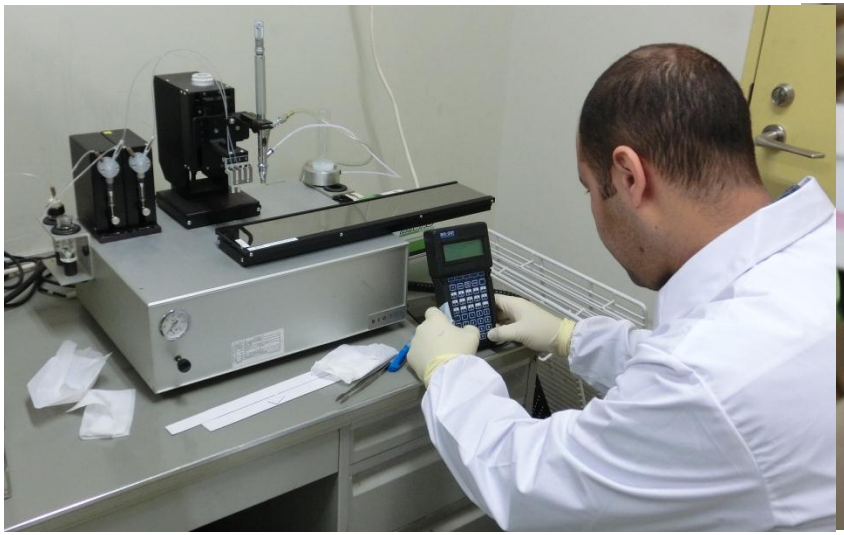
30
300
3000
30000
300000
3000000



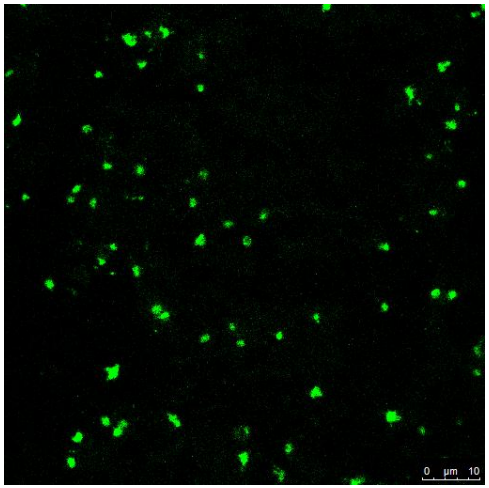
Real Time PCR of Field Genomic sample

Immuno Chromatographic Test (ICT)

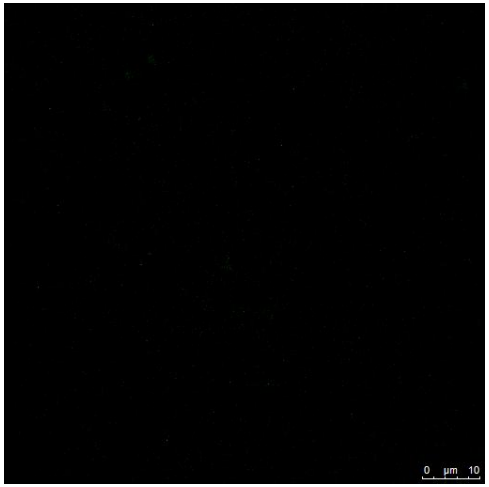
- **We prepared about 400 strips using EMA-2 (t) antigen of *T. equi*.**
- **These ICT strips will be validated on serum samples collected from Indian equine samples.**



IFAT

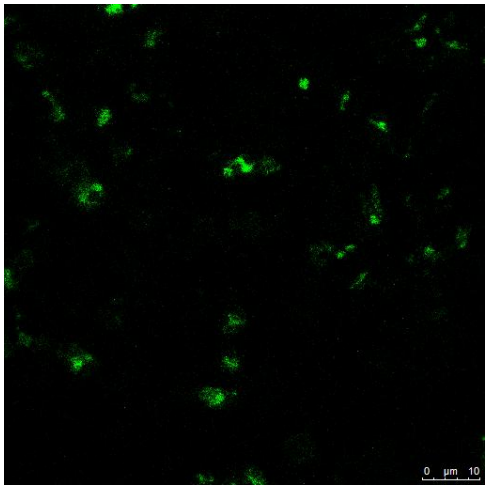


***T. equi* +ve horse**

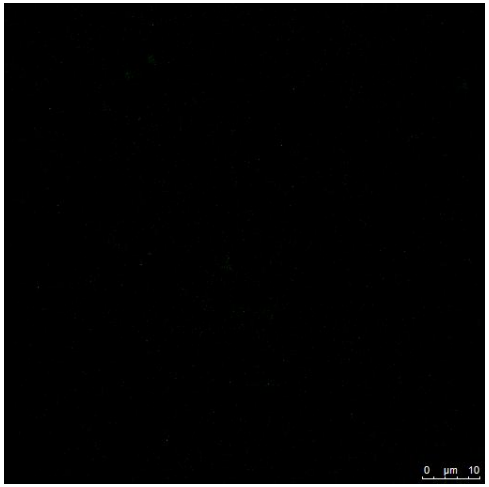


Negative horse serum

Theileria equi



***B.caballi*+ve horse**



Negative horse serum

Babesia caballi

NRCPD Experience

- State-of-art research laboratories equipped with all modern facilities.
- Imparted excellent technical/scientific support for capacity building of NRCE scientists under this OIE Twinning Project

Future Activities

- The ELISA will be optimized using the EMA-1 and EMA-2 expressed antigens.
- The results as obtained in ELISA will be compared with OIE approved CI-ELISA kit, IFAT and in-vitro culture.
- This ELISA will be further transformed in the form of a **diagnostic kit** and validated.
- **qPCR** as a diagnostic test for accessing parasitic load in latently infected equids.


THANKS