Veterinary Parasitology: Regional Studies and Reports 12 (2018) 17–21

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr

Original article

Risk factor analysis associated with *Theileria equi* infected equines in semi-arid and sub-humid ecological enzootic zones of India

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ARTICLE INFO

Keywords:
Theileria equi
Sero-prevalence
India
ELISA
Risk factors

ABSTRACT

Equine piroplasmosis is a haemoprtozoan disease of equines and enzootic in tropical and subtropical countries. A cross-sectional study on sero-prevalence of *Theileria equi*, the causative agent of EP, was performed in semi-arid and sub-humid ecological endemic zones of India including Rajasthan, Haryana and Gujarat states, in order to evaluate the enzootic status/level of exposure to equine population due to this infection and addressed associated risk factors. Serum samples were collected from a total of 1021 equids that comprised of 792 horses, 168 donkeys and 61 mules and evaluated for *T. equi* specific antibodies in equine merozoite antigen-2 (EMA-2) based on indirect ELISA. The state with high sero-prevalence rate was Rajasthan (71.40%), followed by Haryana (60.39%) and Gujarat (48.92%). Overall *T. equi* sero-positivity in equines was 64.44%. Species-wise *T. equi* sero-prevalence was 66.29%, 51.19% and 91.80% in horses, donkeys and mules respectively. The association and risk factor among age, gender and species in relation with *T. equi* infection was statistically analyzed at 95% level of significance ($p < 0.05$). A very high *T. equi* sero-positivity was recorded in 0–1 year age group of equines (60%), indicating that this naïve age group contacts with *T. equi* infected ticks and remains infected throughout its lifetime. The sero-prevalence rate was significantly associated with the species of animal ($p < 0.05$). The risk factor analysis kept mules at higher risk (Odd's ratio; 5.696; 95% confidence interval: 2.25–14.38) of getting infection as compared to horses and donkeys. This study has demonstrated high enzootic nature of *T. equi* infection in semi-arid and sub-humid ecological zones of India. Mules, in comparison to horses and donkeys were found at higher risk of getting *T. equi* infection, indicating that disease prevalence is associated with species of the infected host.

1. Introduction

Ticks and tick-borne diseases have a large impact on animal health and the livelihood of livestock owners, particularly in developing countries. Equine piroplasmosis is a tick-borne disease of equids caused by *Theileria equi* and/or *Babesia caballi* protozoa. About 106 tick species have been reported from India (Ghosh et al., 2007) and ticks of genera *Rhipicephalus* and *Hyalomma* are widely prevalent in > 20 Indian states (Ghosh and Nagar, 2014). *Hyalomma anatolicum anatolicum* tick has been identified as the most important vector tick for transmission of *T. equi* infection in India, which is quite prevalent in semi-arid and sub-humid agro-ecological Indian regions (Malhotra et al., 1978; Kumar and Kumar, 2007; Bhagwan et al., 2015). *T. equi* infects equine erythrocytes and causes acute, sub-acute or chronic disease condition in equids (Mehlhorn and Schein, 1998). *T. equi* infection is responsible for important economic losses to the equine husbandry especially in tropical to temperate zone of the world (Asgarali et al., 2007; Acici et al., 2008). Equines chronically infected with *T. equi* show non-specific clinical symptoms such as fever, depression, icterus, colic and gait incoordination, which make diagnosis of this disease condition difficult (de Waal, 1992). Clinical infection in *T. equi* latently infected equids is not uncommon and often associated with underlined risk factors such as host's age, immunological and concurrent disease infection status, equine farm managemental practices, etc. (Knowles Jr., 1996).

Equine piroplasmosis is an OIE notifiable disease in equines (OIE, 2017). Hence serological testing of this disease is mandatory before transporting equine out of country, in order to prevent the spread of infections to naïve population. India comes under the enzootic zone for this disease condition. Therefore, it is necessary to have time to time updated information on prevalence of disease in different parts of the

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https://doi.org/10.1016/j.vprsr.2018.01.005
Received 17 August 2017; Received in revised form 12 December 2017; Accepted 11 January 2018
Available online 12 January 2018
2405-9390/ © 2018 Published by Elsevier B.V.

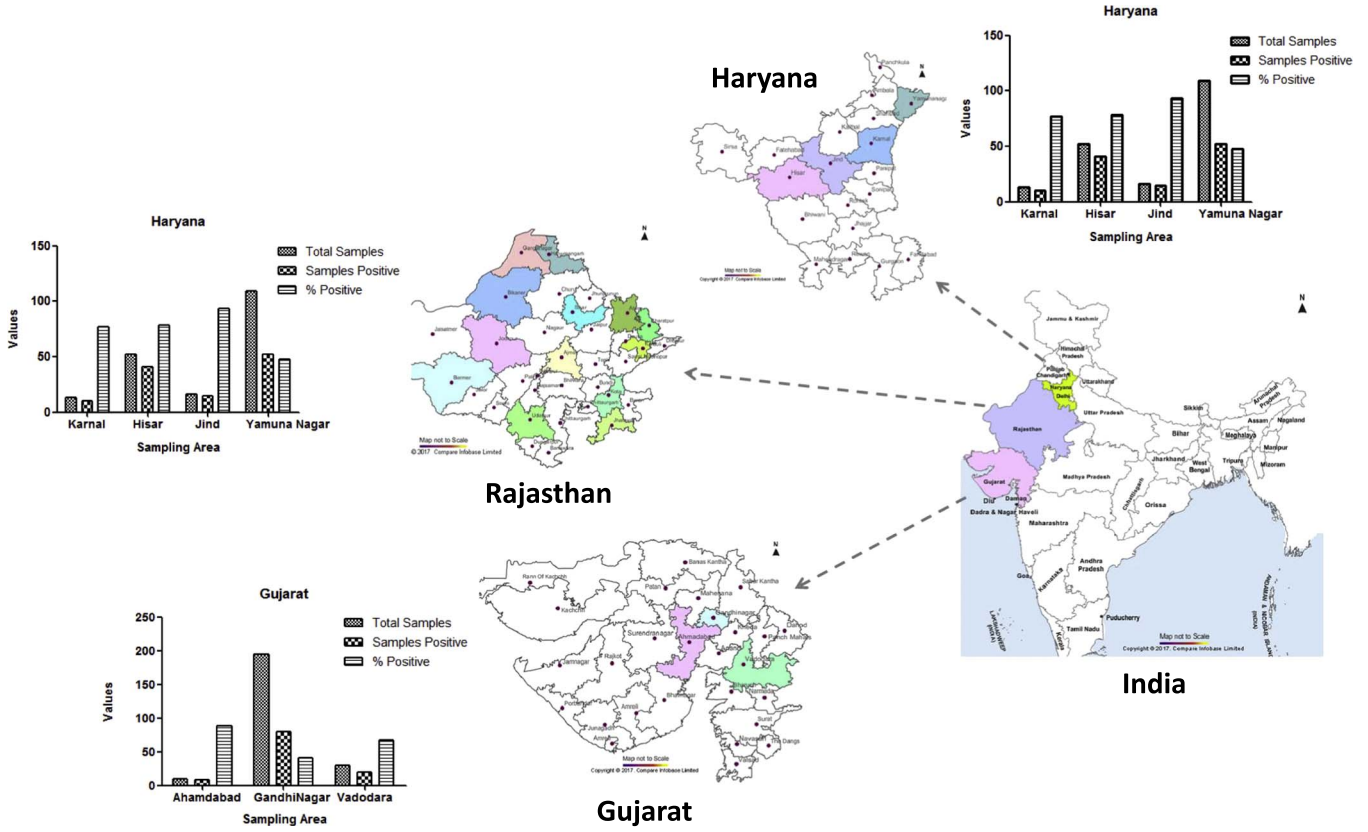

Fig. 1. Geographical distribution of sample collecting area from different Indian ecological zones and graphical representation of total samples collected and *Theileria equi* sero-positivity observed in $_{EMA\text{-}2}$ELISA.

country. Rajasthan, Gujarat and Haryana states altogether harbour 22.88% of the total equine population in India (Livestock Census All India Report-19th, 2012) and most of these equids are donkeys and mules (13.05%), which are reared by the poorest sections of the society (Pal et al., 2013). These Indian states are part of semi-arid and sub-humid agro-ecological regions of India (Gajbhiye and Mandal, 2000). Movement of majority of Indian equine population is un-restricted, which help in contact of naïve equines with *T. equi* infected population. Thus, there was a need to monitor the *T. equi* prevalence rate in different enzootic geographical areas and also to define the associated risk factors, so that disease control strategies can be planned and implemented.

2. Materials and methods

2.1. Sampling area

This study involved the equine population in north-western region of India encompassing Haryana, Rajasthan and Gujarat states covering geographical area between 25°25′ to 30°30′N latitude to 68°32′ to 77°22′E longitudes as represented in the map (Fig. 1). The representative equine blood samples were collected from twenty different locations in Haryana, Rajasthan and Gujarat states (Fig. 1). The sample size required for comparing proportions was calculated according to Thrusfield, 2005. Sample size in these geographic area was defined on expected *T. equi* sero-prevalence of 32% (Kumar et al., 2013) on finite equine population (Rajasthan = 119,244; Gujarat = 57,098 and Haryana = 39,558) with a confidence interval (CI) of 95% and absolute precision of 5%. These sample size criteria were selected to maximize the accuracy of prevalence and risk factor analysis. Based on these criteria total 1002 equine population samples were required. In total 1021 samples were collected from horse, mule and donkey population belonging to twenty different locations of three states, as above. All the blood samples were collected (without anti-coagulant) from equines during the year 2013–2015. The serum was obtained by centrifuging the sample for 15 min at 800*g* and stored at − 20 °C until further use.

A questionnaire was used to collect information on each sampled equid. Data on various associated risk factors were gathered at location site during the sample collection. The risk factors included in the above questionnaire were as follows - type of equine species (horse/donkey/mule), age groups (0–1 years, 1–5 years, 5–10 years, > 10 years), gender (male/female) and management practices (treatment against ectoparasites, type of housing, sanitary and deworming practices).

2.2. EMA-2 enzyme linked immunosorbent assay ($_{EMA\text{-}2}$ELISA)

The serum samples from these equids were tested in $_{EMA\text{-}2}$ELISA at 1:200 dilution and OD_{492} values were recorded. The $_{EMA\text{-}2}$ELISA cut-off point was determined by calculating the relative percent positivity (RPP) of each test sample using the following formula (Kumar et al., 2013).

$$\text{RPP} = \frac{OD_{492}\text{ of tested sample} - OD_{492}\text{ of negative control sample}}{OD_{492}\text{ of positive control sample} - OD_{492}\text{ of negative control sample}} \times 100$$

The cut-off RPP value for considering the positive reaction of a sample in $_{EMA\text{-}2}$ELISA was 22 (Bhagwan et al., 2015; Kumar et al., 2015) and serum sample showing RPP value above cut-off was considered serologically positive.

2.3. Statistical analysis

The association of prevalence of *T. equi* by $_{EMA\text{-}2}$ELISA with various

geographic areas of three states (Haryana, Rajasthan and Gujarat) and epidemiological risk factors in this study were statistically analyzed by Pearson's *chi*-square test $p \leq 0.05$. Risk factor analysis and other statistical tests were done by SPSS software version 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.).

3. Results and discussion

In our previous study, EMA-2 recombinant based ELISA was developed (Kumar et al., 2013) and applied to sero-prevalence studies on Indian equine population. Haryana, Rajasthan and Gujarat are the three adjoining states expanding from trans-gangetic plain to western dry to Gujarat plain and hill regions. These three states are highly *T. equi* enzootic geographical regions (Kumar et al., 2013) and have been selected for the present study. Previously we recorded overall 32.65% sero-prevalence of *T. equi*, and this criterion was applied in the present study to estimate the sample size. The finite number of equine population was considered to calculate the total required sample size. Serum samples were collected from twenty different areas in Gujarat (3), Rajasthan (13) and Haryana (4) regions (Fig. 1). Sumbria et al. (2016) and Chilundo et al. (2017) have also applied the same methodology while studying spatial distribution and prevalence of *T. equi* in equines and ecto-parasitic infection in pigs, respectively.

In this study, 658 (64.44%) serum samples out of total 1021 equidae samples were found positive for *T. equi* infection. The RPP values of these positive samples were > 22 (Fig. 2). The per cent RPP values of Haryana, Rajasthan and Gujarat samples ranged from 26 to 123; 25.3–157.5 and 23.07–138.46, respectively (Fig. 2). One hundred seventeen (61.5%) out of 190 samples from Haryana, 427 (71.40%) out of 598 samples from Rajasthan and 114 (51.12%) out of 233 samples from Gujarat were detected sero-positive in $_{EMA-2}$ELISA (Table 1). The sero-positivity among these three states differ significantly ($p \leq 0.05$). The climate of Haryana, Rajasthan and Gujarat is sub-tropical semi-arid/arid to sub-humid type, most congenial to the breeding of ticks and transmission of tick-borne diseases (Geevarghese et al., 1997; Rehman et al., 2017). The variations in sero-prevalence rates of *T. equi* in this study among different enzootic areas may be due to these factors, which may require further thorough systematic investigations.

Sero-reactivity of *T equi* antibody was also analyzed in different category of equids on the basis of species, age, and gender (Table 1). It has been observed that horses, mules and donkeys from Rajasthan were more infected with *T. equi* as compared to other areas (Table 1). As per Livestock Census All India Report-19th, 2012, Rajasthan has the maximum number of horse and pony, mule and donkey population (55.23%) among these three states under this study. This may be perpetuating factor for more *T. equi* infected equine species in Rajasthan state. It was interesting to record 60% *T. equi* sero-prevalence in 0–1 year age group of equines, indicating that this naïve age group

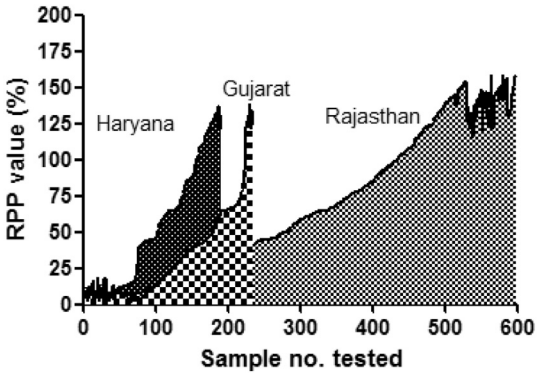

Fig. 2. Relative per cent positivity (RPP) of 1021 field serum samples in $_{EMA-2}$ELISA. RPP of 658 positive serum samples ranged from 23.07 to 157.5, while cut off was 22.

contacts with infected ticks at early age and remains infected throughout its lifetime. Kumar et al. (2008) reported that new-born foals are born naive and theirs passively transferred immunity is transitory, which wanes after 63 to 77 days after foaling; consequently foals becomes susceptible to natural *T. equi* infection. A very high incidence of *T. equi* infection (50.35% to 71.24%) has been recorded in female population (Table 1); so it was but natural that these young foals (0–1 year's age) may get infected from the *T. equi* infected ticks, dropped-off from their infected dam. *Hyalomma a. a.* transmit *T. equi* sporozoites transtiadelly, hence physical contact of naïve foals/equids with infected ticks is mandatory (Kumar et al., 2007).

The sero-prevalence rate and associated risk factors of *T. equi* with respect to species difference, age, and the gender of natural hosts were statistically analyzed on total sample data and detailed in Table 2. The prevalence rate was higher (91.80%) in mule population as compared with horse (66.29%) and donkey (51.19%). There is a phenomenal increase of 43.34% in mule population in India, whereas donkey's population decreased by 27.22% over the previous livestock census of 2007 (Livestock Census All India Report-18th, 2007). Increasing preference of the Indian equine owners towards mules as compared to donkey may be a major cause for higher sero-prevalence of *T. equi* infection in mules among equid population. The *chi* square value (34.024, $p \leq 0.05$) indicated significant association of *T. equi* infection with species difference. The mules were 5.696 (OR value range: 2.25–14.38) times more at risk of getting *T. equi* infection than horses. Mules are generally reared by the poor section of a particular community under unhygienic conditions (Pal and Legha, 2008), which may increase the chances to contact with infected tick vectors (Sumbria et al., 2016; Bhagwan et al., 2015; Kouam et al., 2010). The high prevalence rate of *T. equi* in mules has been reported from South Spain (66.21%; García-Bocanegra et al., 2013), Brazil (83.30%; Santos et al., 2011), Jordan (75%; Qablan et al., 2013) and Greece (76.9%; Kouam et al., 2010).

The overall sero-prevalence of *T equi* infection in equines was not affected in relation with age groups and gender of hosts (Table 2). The sero-positivity of *T. equi* among different age groups (0 to 10 years and geriatric) varied from 58.12% to 66.39% (CI, 95%). These findings are in accordance with previous observations (Moretti et al., 2010; Shkap et al., 1998; Sigg et al., 2010; Acici et al., 2008; Karatepe et al., 2009). The non-significant difference in the sero-positivity in different equine age groups signifies the fact of lifetime carrier status of *T. equi* sero-positive equids (Mehlhorn and Schein, 1998). Risk factor analysis on the pooled sample data (Table 2) showed statistical non-significant difference ($p < 0.01\%$) for *T. equi* sero-prevalence among male and female population. These findings are in accordance to other studies (Karatepe et al., 2009; Sigg et al., 2010; Hussain et al., 2014; Montes Cortés et al., 2017). Our study group mainly included equids reared by poor farmers, who use these animals for carting and carriage to earn their daily livelihood. They maintain male and female equids without any discrimination; therefore *T. equi* incidence differed non-significantly.

4. Conclusions

The high sero-prevalence of *T. equi* antibodies (51.12% to 71.4%) among Indian semi-arid and sub-humid ecological zones, suggested endemicity of this infection. Rajasthan state has high enzootic areas for this disease condition followed by Haryana and Gujarat states. *T. equi* sero-positivity within different geographic areas was largely not affected by demographic risk factors i.e. age and sex. A very *T. equi* sero-positivity was recorded in 0–1 year age group of equines (60%), indicating that this naïve age group contacts with infected ticks and remains infected throughout its lifetime. Mules, in comparison to horses and donkeys were found to be at higher risk of contact *T. equi* infection, indicating that disease prevalence is associated with species of the infected host. These findings are more useful for small and marginal equine keepers of these ecological zones and may help them to plan

Table 1
Sero-prevalence of *Theileria equi* in three endemic states of India indicating positivity in different categories of equines.

Category	Haryana (n = 190)			Rajasthan (n = 598)			Gujarat (n = 233)		
	Samples tested (positive/total samples tested)	% Sero-prevalence	CI*	Samples tested (positive/total samples tested)	% Sero-prevalence	CI*	Samples tested (positive/total samples tested)	% Sero-prevalence	CI*
Type of animal									
Horse	87/151	57.61	49.73–65.49	379/540	70.18	66.32–74.04	59/101	58.41	48.8–68.02
Donkey	3/5	60.00	17.08–102.92	26/31	83.87	70.92–96.32	55/132	41.66	33.25–50.07
Mule	27/34	79.41	65.82–93	22/27	81.48	66.83–96.13	0	NA	NA
Age groups (in years)									
0–1	3/5	60.00	17.08–102.92	41/68	60.29	48.66–71.92	0	NA	NA
1–5	46/78	58.97	48.05–69.89	162/225	72.00	66.13–77.83	35/63	55.55	43.28–67.82
5–10	49/80	61.25	50.57–71.93	181/247	73.27	67.75–78.79	73/138	52.89	44.56–61.22
> 10	19/27	70.37	53.15–87.59	43/58	74.13	62.86–85.4	6/32	18.75	5.23–32.27
Gender									
Male	85/145	58.62	50.6–66.64	147/205	71.70	65.53–77.87	43/92	46.73	36.53–56.93
Female	32/45	71.11	57.87–84.35	280/393	71.24	66.76–75.72	71/141	50.35	42.1–58.6
	117/190	61.5[a]		427/598	71.4[b]		114/223	51.12[c]	

a,b,c: % sero-prevalence among Haryana, Rajasthan and Gujarat states differ significantly at $p \leq 0.05$.
* Confidence interval (CI) at 95%.

Table 2
Risk factor analysis on different categories of equids sampled from ecological *Theileria equi* endemic zones of India.

Category	Total samples data (n = 1021)					Odd ratio (OR)	
	Samples tested (positive/total samples tested)	% prevalence	CI[a]	Chi square value	Relative Risk factor	OR values	CI[a]
Type of animal							
Horse	525/792	66.29	65.49–86.70	34.024[#]	a	a	
Donkey	86/168	51.19	55.76–101.07		0.772	0.533	0.381–0.747
Mule	56/61	91.80	37.13–103.80		1.385	5.696	2.25–14.38
Age groups (in years)							
0–1	44/73	60.27	49.68–92.20	3.308	a	a	
1–5	243/366	66.39	66.50–88.05		1.102	1.302	0.77–2.18
5–10	303/465	65.16	73.14–91.41		1.081	1.233	0.74–2.04
> 10	68/117	58.12	67.29–97.38		0.964	0.915	0.50–1.65
Gender							
Male	275/442	62.22	45.27–71.69	1.691	a	a	
Female	383/579	66.15	81.78–100.23		1.063	1.187	0.91–1.53

[a] 95% confidence interval.
[#] Significant difference at $p \leq 0.05$.

disease control strategies.

Conflict of interest statement

The authors of this research paper declare that they have no conflict of interest.

Acknowledgements

Authors wish to acknowledge their gratitude to the Director, ICAR-National Research Centre on Equines (Indian Council of Agricultural Research), Hisar, Haryana, India for providing all the necessary facilities for conducting this study. They would also like to thank Mr. Joginder Singh and Mr. Ajmer Singh for technical assistance provided during the experiments.

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