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Molecular characterization and pathogen detection in *Amblyomma gervaisi* and *Amblyomma varanense* collected from snakes of India

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Abstract:

The main objective of this study was to underscore detail gross and ultra-structural morphology, molecular characterization of ticks found on snakes and their ability to carry zoonotic pathogens. The investigation was carried out throughout the North-Eastern states of India during different seasons from March, 2020 to February, 2021. The outer surface of different species of snakes that were either kept in zoos or captured or killed by local people were thoroughly examined for presence of any ticks. Some of the collected ticks were preserved in absolute alcohol for molecular study while rest was kept in 70% alcohol for Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Light microscopy, SEM and TEM revealed specific morphological features through which the collected ticks were identified as *Amblyomma gervaisi* and *Amblyomma varanense* and further confirmation was revalidated by molecular detection. The ticks were commonly found during the rainy season (July to October) and Indian

black cobra (*Naja naja*) was found more commonly infested with both genera than that of other three species of snakes. The existence of tick-associated pathogens in *A. gervaisi* and *A. varanense* were determined by molecular and bioinformatics techniques. DNA isolated from snake ticks yielded products by the tick mitochondrial 16S rRNA PCR. Sequence homology and phylogenetic analysis of amplified PCR product revealed that the pathogen infecting *Amblyomma* ticks was a member of the genus *Coxiella*. The partial sequence of the *Coxiella* agent of *A. gervaisi* and *A. varanense* grouped with *Coxiella* symbionts of other hard tick species (Ixodidae). Additionally, present study suggests a very long period of coevolution between ticks and *Coxiella* symbionts and suggests that the original infection might have occurred in an ancestor common to the 2 main tick families, Argasidae (soft ticks) and Ixodidae (hard ticks). However, further extensive study of additional tick species and expanded host populations must be required to conclude this evolutionary relationship between ticks and *Coxiella* symbionts.

Key words: *A. gervaisi*; *A. varanense*; morphology; SEM & TEM; PCR; *Coxiella burnetii*; phylogenetic.

1. Introduction

Reptiles have a significant role in our ecosystem. Various species of ticks are commonly found in reptiles including snakes but the intensity of infection and the effects on their reptilian hosts are not thoroughly studied [1]. Although ticks rarely occur in large numbers to cause either severe blood loss or other direct injuries to snakes but they can transmit various diseases to animals and humans [2, 3, 4]. With the increasing international trade of wild animals, there is every chance of high risk of disseminating exotic ticks and the associated pathogens with them [5, 6]. During the course of evolution of ticks, the first pair of limbs is modified to form chelicerae for piercing into the skin of their hosts and the second pair is transformed to form palps. On the other hand, third to sixth pair remained as the actual legs for walking. *A. gervaisi* and *A. varanense* ticks are exclusively found on reptiles. Among these two species of ticks,*A. varanense* is most widely distributed in various countries including India [7].

It is reported that reptiles including snakes carry various types of parasites and ticks for a considerable period of time without any harmful effect unless other factors undermine their immunity and then allow the disease to flare up [8].Ticks are considered more successful vectors

for transmitting various pathogens than any other blood sucking arthropods[9]. The rainforest area of North Eastern region of India along with its suitable environment and climatic condition favour the parasitism of snakes by ticks. *A. varanense* utilizes monitor lizards and large snakes as primary hosts although it also infests chelonians and mammals [3]. It has the ability to spread outside its geographical range to infest new reptile hosts. It has characteristic metallic yellowish green markings with reddish-brown scutum [7]. However, some specimens are in-ornate making difficulties in identification.The tick, commonly known as the 'Asian monitor lizard tick' is a potential vector of spotted fever group rickettsiae [10]. *A. gervaisi* is a hard-bodied tick belonging to the genus *Amblyomma*. It infests various species of snakes and monitor lizards in Asia and Asia-minor [11]. Morphologically it shares the same characters of the genus *Amblyomma*. Previous studies have reported that it is a potential vector of *Coxiella burnetii*[10].

Hard and soft ticks are the most important arthropods to be naturally infected with *C. burnetii* 12]. At least 40 species of ticks have been found to be infected with *C. burnetii*. Ticks become infected during blood feeding from infected animals and then transmit the bacterium to other animals during the next blood meal or spread by air through dried tick faeces. Multiplication of *C. burnetii* occurs in the mid gut cells of ticks. In some ticks, both transtadial and transovarian transmission of *C. burnetii* take place. Ticks also spread the pathogen by contaminating the environment through their faeces, saliva and coxal fluid but there is no evidence that transmission of *C. burnetii* to human being occurs by ticks [13]. Human infection mostly takes place after inhalation of contaminated air, consumption of dairy products or contact with infected animals [14].

Earlier, most of our taxonomical knowledge of ticks was based on morphological studies under light microscope. However, with the advent of SEM and TEM and various molecular techniques, it is now possible to observe the diagnostically significant taxonomic morphological features in more detail and assist us to accurately identify the species. Several studies have shown that molecular data together with morphology is very useful for species identification. Mitochondrial and ribosomal RNA spacer sequences II are increasingly being used to investigate molecular identity of species. Some studies on SEM and TEM of reptilian ticks have been carried [14,15,16].

Keeping this view in mind, the present study has been undertaken to morphologically and molecularly characterize of snake ticks (*A.gervaisi* and *A. varanense)*as well as molecular detection of endopathogen of ticks collected from different snakes belonging to the families of pythonidae, viperidae, elapidae and colubridae.

2. Material and methods

2.1 Place of study

The ticks were collected from four species of snakes from different parts of North eastern region of India from March, 2020 to Feb, 2021. The snakes were examined in different zoos as well as locally captured/run over by vehicle/or killed by people. A total of 765 snakes belonging to the families of Pythonidae (*Python molurus molurus*), Viperidae (*Vipera russelli*), Elapidae (*Naja naja*) and Colubridae (*Ptyas mucosa*) were examined.

2.2 Morphological study and laboratory rearing

Live ticks were brought to the laboratory and observed under stereo-zoommicroscope (Euromex, Holland)and taxonomically identified [17]. One male and one female tick from each species were processed and permanent slides were made. For further surface morphology and internalstructuresin detail, ticks were further processed for SEM and TEM.

Different developmental stages of ticks' *i.e.*17 nymphs, 110 females and 58 males of *Amblyomma* were separated. All 17 nymphs and 20 females were separated for molecular analyses (described below), and the remaining adults were used to form a laboratory colony.Ticks were fed on tick-naïve rabbits; then engorged ticks were transferred to an incubator and maintained at 25°C and 85% relative humidity.

2.3 Preparation of samples for SEM and TEM

Any extracellular debris like mucus, blood or other body fluids and tissue fragments which otherwise make difficult for ultra structural observation were removed carefully by washing the ticks several times with distilled water with the help of a camel brush. The samples were then fixed in 2.5% glutaryldehyde at a temperature of 4ºC for 24 hours. The fixed samples were again washed three times in phosphate buffer saline (pH - 7.2) and then in distilled water followed by

acetone dehydration. After acetone dehydration, the specimen were dried with liquid carbon dioxide at its critical point *i.e.* 31.5ºC at 1100 psi. They were then dipped in tetra methyl saline for 5-10 minutes with two changes at 4ºC. They were then brought to room temperature (25-26ºC) for drying. Finally, the samples were mounted on aluminum stubs and were gold coated in a sputter coat. Images were capture using SEM (JSM-6360-JEOL) and TEM (JEM-2100-JEOL).

2.4 Isolation of genomic DNA from ticks and eggs

DNA was extracted from ticks by the guanidineiso-thiocynate-phenol technique [18] using a commercial kit (DNeasy® Blood and Tissue Kit, Qiagen) following manufacturers' protocol with slight modifications. Briefly, individual tick was triturated with Tris-HCl buffer (Invitrogen, USA) in a well sterilized pestle and mortar and the whole material was transferred in an 1.5 ml micro-centrifuge tube and incubated at 56ºC after adding Proteinase-k (20mg/ml) for complete lysis. After adding ethanol (96-100%)the mixture was transferred into a spin column and centrifuged at 10,000 rpm for 2 minutes. After washing with wash buffer twice, the elution buffer was added to the column and incubated for 2 minutes at room temperature. Finally, the column was centrifuged and centrifuged material was kept at -20ºC for downstream use.

The extracted DNA was tested by a battery of PCR assays targeting bacterial endosymbionts of the genera *Rickettsia*, *Borrelia* and *Coxiella*. PCR was performed with genus-specific primers shown in Table 1. In each PCR assay, negative controls (no template DNA) and an appropriate positive control sample (DNA of *Rickettsia rickettsiae, Borrelia burgdorferi, C. burnetii*) were run together along with the tick DNA samples.Twenty egg pools (each containing 20 eggs) derived from 20 different females, and 10 individual eggs were taken from the *Amblyomma* colony and subjected to DNA extraction as described above. These samples were tested by PCR targeting the pathogen genes listed in Table 1.

Table 1: List of primers used in this present study

Parasite (Target gene)	Primers (5' – 3')		Amplicon size (bp)	Annealing temperature (°C)	References
	Forward	Reverse			

Amblyomma gervaisi (18S rRNA)	ATTTTGACTATAC AAAGGTATTG	AGAAATATCCTAAT CCAACATC	253	55	Designed from published sequence available at NCBI
Amblyomma varanense (18S rRNA)	AATTAAGGACAAG AAGACCC	AGAAATATCCTAAT CCAACATC	253		
Rickettsia spp. (*gltA*)	GCAAGTATCGGTG AGGATGTAAT	GCTTCCTTAAAATTC AATAAATCAGGAT	401	50	[19]
Borrelia spp. (*fla*)	ACATATTCAGATG CAGACAGAGGT	GCAATCATAGCCAT TGCAGATTGT	665	60	[20]
Coxiella spp. (16S rRNA)	GGGGAAGAAAGTC TCAAGGGTAATAT CCTT	TGCATCGAATTAAA CCACATGCTCCACC GC	532	58	[21]

The PCR reaction was carried out in 25µl of 1X PCR green buffer (Thermo Scientific, USA) containing 1 unit of DreamTaq DNA polymerase, 10 pmol of each primer and 0.2 mM concentration of each deoxyribonulceotide triphosphate and 50ng of template DNA. Amplification was performed using a C1000 thermal cycler (BioRad, USA) under following conditions: initial denaturation at 94°C for 5 min, followed by 35 amplification cycles (94°C for 30 sec, 50-60°C for 45 sec and 72°C for 1 min) and a final extension at 72°C for 10 min (Annealing temperature for different genes are shown in Table 1). The PCR amplicons were analysed by 1.5% (w/v) low melting agarose gel electrophoresis and positive PCR products were extracted and purified using Qiaex II gel extraction kit (Qiagen, Germany) following manufacturers protocol.

2.5 Molecular cloning and sequencing

The purified PCR products of the desired gene of the ticks and endosymbionts from each sample was separately ligated into pTZ57R/T TA cloning vector (Thermo Scientific, USA) and incubated at 4°C for overnight. The plasmid DNA constructs were transformed into competent DH5α *Escherichia coli* cells using InsTAclone PCR cloning kit (Thermo Scientific, USA) according to manufacturer's protocol. The transformed cells were plated immediately on pre-warmed LB agar plates supplemented with ampicillin (100µg/ml), X-gal (30µg/ml) and IPTG (0.5mM/ml) for the development of blue white colonies. The positive clones were confirmed by

colony PCR using specific primers. The stab cultures of two positive clones per sample containing the desired gene were custom sequenced from the Department of Biochemistry, Delhi University, South Campus, India. The fragments were sequenced at least twice to reduce possibility of sequencing errors.

2.6 Sequence analysis

All the newly generated sequences of the ticks and endosymbiont were compared with each other and with published sequences in the nucleotide database in GenBank by BLAST programme of the National Centre for Biotechnology Information and aligned by ClustalW.

2.7 Phylogenetic analysis

Phylogenetic analysis was done independently with the sequences of desired gene fragment by using the MEGA6.0 software with the maximum likelihood method. The nucleotide substitution model for best fit to the data set was evaluated in MEGA6.0. Tamura-Nei model (TN93) with gamma distribution (TN93+G) and Kimura-2 was found to be the model of choice for phylogenetic analysis. The phylogeny was analyzed using 1000 bootstrap replications [22].

2.8 Prevalence study

The prevalence of *A.gervaisi* and *A. varanense* was recorded according to the species of snakes. The prevalence (P) was estimated according to standard method [23] and by the formula as given below:

$$P = \frac{\text{No. of infected snakes during specified period}}{\text{Total snakes surveyed}} \times 100$$

2.9 Statistical analysis

The epidemiological data were subjected to Chi-square (χ^2) test to assess if there was a statistically significant association in tick infestation in the various groups [24].

2.10 Ethical statement

All animal experiments were carried out strictly as per the guidelines issued by ARRIVE and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)

and was approved by Institutional Animal Ethics Committee with reference No. CAU/CVSc/IAEC/no. 6689, dtd, Selesih, the 27th, December, 2019.

3. Results

A total of 185 numbers of ticks was collected from four snake species during the whole study period (Table 2). Collected ticks were found as nymphs and semi-engorged or fully engorged during collection. Ticks collected from Viperidae (*Vipera russelli*), Elapidae (*Naja naja*), Colubridae (*Ptyas mucosa*) and Pythonidae (*Python molurus molurus*) were taxonomically identified as *A. gervaisi* and *A.varanense* (*A.gervaisi*– 11 nymphs, 65 female and 21 male; *A. varanense*–6 nymphs, 45 female and 37 male).

Table 2: Prevalence of ticks according to the species of snakes

Species of snakes	No. of snakes examined	No. of snakes found positive	Prevalence %
Python molurus molurus	128	38	29.68
Naja naja	249	89	35.75
Vipera russelli	108	22	20.37
Ptyas mucosa	280	72	25.71

Taxonomic position

Phylum	Arthropoda
Class	Arachnida
Order	Acarina
Sub order	Ixodoidea
Family	Ixodidae
Genus	*Amblyomma*
Species	*gervaisi*
	varanense

PCR revealed a positive amplification of 253bp for *A. gervaisi* and *A. varanense* (Fig. 1A). The sequence analysis of amplified fragments of tick 18S rRNA gene (253bp) allowed us to revalidate the tick species as *A.gervaisi* and *A. Varanense* along with morphological details.

Ticks were found in more numbers in Indian black cobra (*Naja naja*) than other three species of snakes and highest incidence of ticks were noticed during monsoon season (Table 2 and 3).

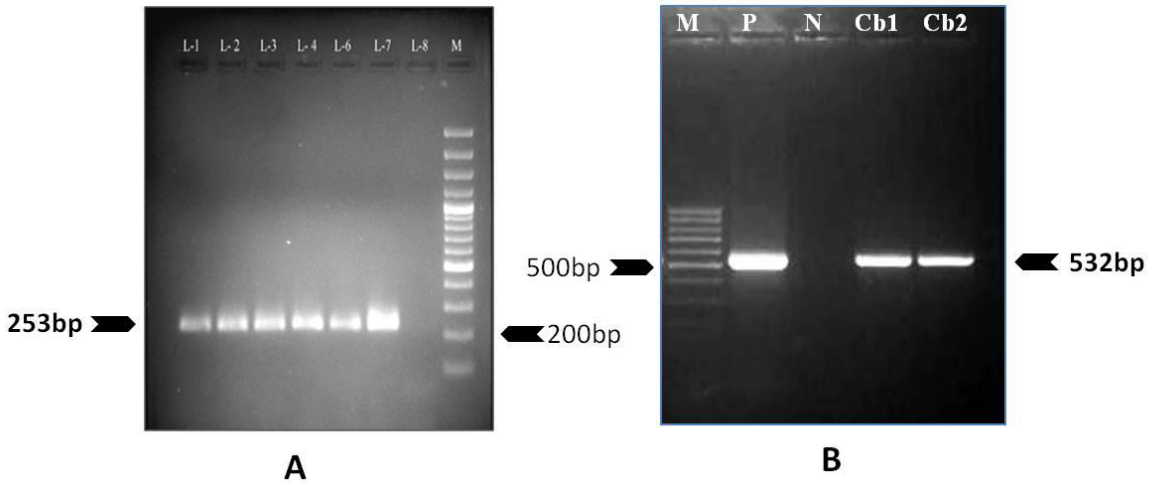

Figure 1: (A) PCR amplification of 18S rRNA gene fragment of *Amblyomma gervaisi* and *A. varanense* in 1.5% (w/v) agarose gel: Lane 1-3 – Positive amplification (253bp) of *A. gervaisi*; Lane 4-7 – Positive amplification (253bp) of *A. varanense;* Lane 8 – No template control; M – 100bp plus DNA ladder. (B) PCR amplification of 16S rRNA gene fragment of *Coxiella burnetii* endosymbiont of *Amblyomma* ticks in 1.5% (w/v) agarose gel: M – 100bp DNA ladder, P – Positive control, N – No template control, Cb1 – Positive amplification (532bp) from *A. gervaisi* ticks, Cb2 - Positive amplification (542bp) from *A. varanense* ticks.

Table 3: Sex-wise and seasonal prevalence of ticks

Particulars	No. of snakes examined	No. of snakes found positive	Prevalence %
Sex			
Female	525	165	31.43
Male	240	56	23.34
Chi-square ($\chi 2$) value : 5.2541* (p-value < 0.05)			
Season			
Summer (April – June)	240	61	25.42
Monsoon (July – October)	318	107	33.65
Winter (November – March)	207	53	25.60
Chi-square ($\chi 2$) value : 6.0016* (p-value < 0.05)			

PCR revealed a positive amplification of 532bp for *C. burnetii* (Fig. 1B). All 37 ticks (17 nymphs, 20 females) and all egg samples (20 pools and 10 individual eggs) yielded PCR products for the 16S rRNA gene of*Coxiella*. From them two isolates (VPA/Tick/Cb1 & VPA/Tick/Cb2) from each tick genera were taken for further analysis.

3.1 *Amblyomma gervaisi* under SEM

Both male and female ticks looked like a tear drop. The mouth parts were long and well visible from dorsal aspect. The basis capitulumwas flask shaped in both male and female. Both sexes were dorso-ventrally flattened and the posterior extremity was wider than the anterior part of the body. Numerous pores were visible on the dorsal surface. The chelicerae showed distinct mandibles digits. The unpaired hypostome was spatula in shape and the denticleswas arranged in definite shape. Each tooth was terminated distantly in crown or corona. The hypostome was also showed a median toothless line running along the ventral surface. The dental formula was 3/3. Two extended pulps were originated from each side of the base of the hypostome and each consisted of four articles. Tip of the palp was well organized and a round head like structure with densely packed sensory bristles projected outward from a comma shaped depression. Ventral surface of the male was found armed with two pairs and one unpaired ventral plates (Figure 2A-F)

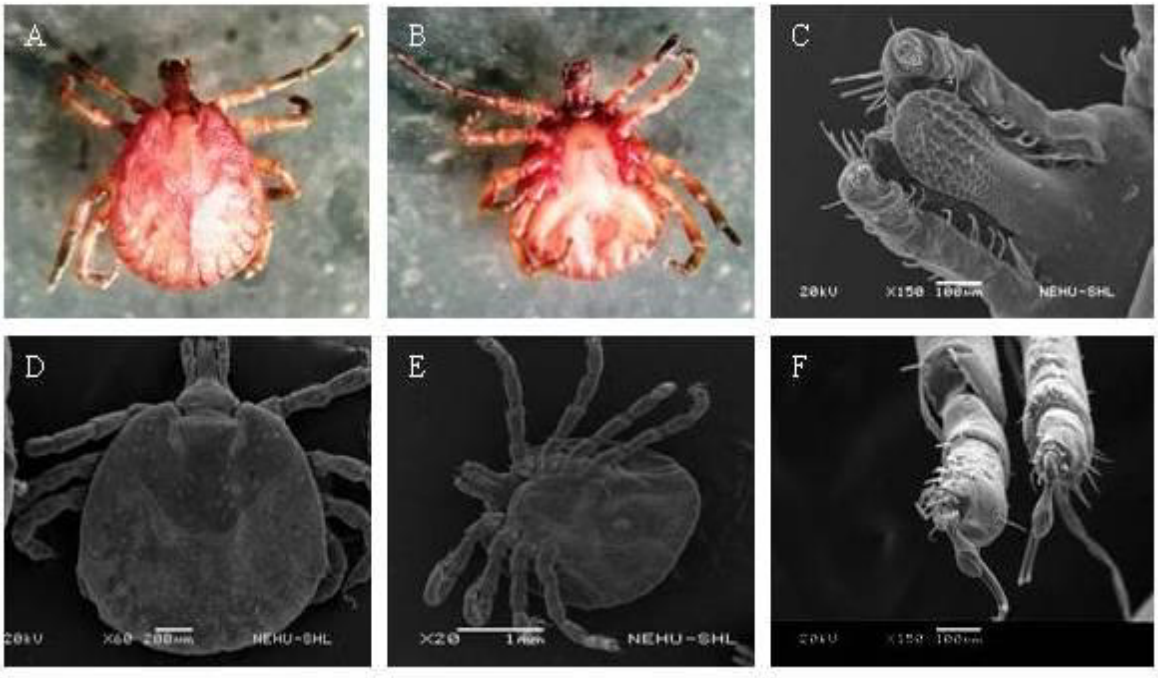

Figure 2: *Amblyomma gervaisi*. A –Dorsal part under stereozoom microscope; B: Ventral part under stereozoom microscope; C: Mouth part of adult tickshowing 3/3 arragement of teeths; D: Dorsal part showing scutum, numerous pores and two porose areas; E: Accessory shields, genital plates and anus; F: Sensory bristles and comma shaped hook like projection on 1st left leg

3.2 *Amblyomma varanense* under SEM

Both male and female of this species were dorsoventrally flattened with numerous randomly distributed spines. One pair of cervical groove was visible behind the rectangular shaped basis capitulum.A pair of ventral plates was present on the ventral surface of male. The surface of the anal plate was armed with 4-6 rows of symmetrically arranged spines and the anal groove surrounded the anus posterior. The coronal surface of the hypostome was smooth and the denticles were arranged symmetrically. The dental formula was 4/4 and several small teeth were found united together at the base just like a scale. One pair of distinctly margined and comma shaped spiracles was found behind the fourth coxa. Four pairs legs of were attached at the ventro-lateral aspect of the body and each consisted of seven segments. Each tarsus was provided with a pair of curved claws. The genital organ was located in the mid ventral surface immediately behind the basis capitulum (Figure 3A-F)

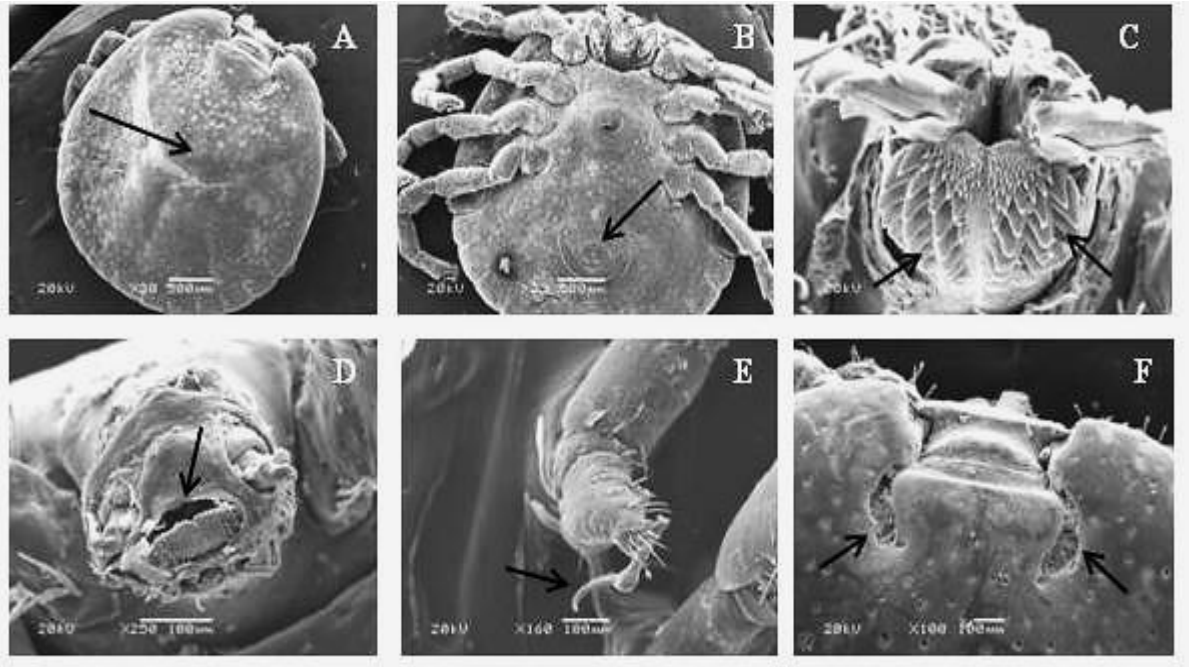

Figure 3: *Amblyomma varanense*. A: Dorsal surface of adult tick; B – Ventral surface of adult tick showing unarmed genital pore and anal groove; C: Mouth part showing 4/4 arrangements of teeth; D: Mouth part and basis capitulum; E: Densely packed sensory bristles and a comma like spiny projection on leg; F: Flask shaped basis capitulum and numerous pores on the surface

3.3 *A. gervaisi* and *A. varanense* under TEM

The integument of both *A.gervaisi* and *A.varanense* was investigated under TEM. The study revealed an outer epi cuticle and an inner procuticle. The epi cuticle comprised of wax, cuticulin and protein epicuticle layer. The wax layer showed numerous crater-like deposits, oval or circular discs and numerous folding. The procuticleconsisted an exo-,endo- and a subcuticular layer. Underlying the cuticle, flattened epidermal cells were connected via desmosomes and contained rough endoplasmic reticulum, ribosome, mitochondria etc. Scattered dermal glands were located beneath the cuticle and were continuous through dermal ducts and surface pores (Figure 4A-C).

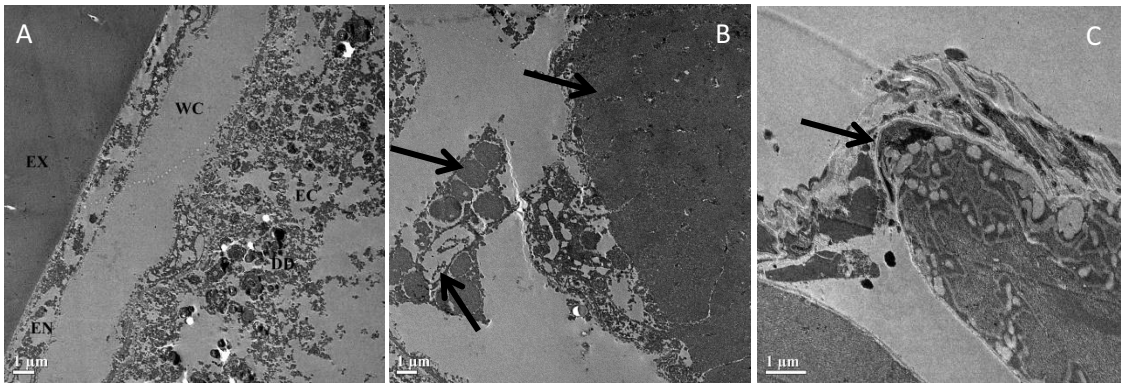

Figure 4: A: Ultrastructure of integument of adult tick (EX: exocuticle; EN: endocuticle; WC: wax canal; EC: Epithelial cells; DD: Dense discs); B: Endocuticle and cuticular lumen; C: Microtubules, Rough endoplasmic reticulum and lysosomal vesicles

3.4 Sequence analysis

A sequence of amplified fragments of tick 18S rRNA allowed to identify tick species as *A.gervaisi* and *A.varanense*. The 253bp of amplified DNA from both ticks revealed that their sequences were identicaland submitted to the GenBank under the accession no. MK256949 and MK256950. NCBI-BLAST search exhibited the closest match to the sequence submitted from Kerala, South India (MG888733) with 100% identity USA (MH282856) with 86.6% (Figure 5) respectively. Sequences of the 16S rRNA (~532 bp) of *C. burnetii*obtained in the present study were submitted to GenBank for obtaining accession number. Sequence similarity searches in BLAST showed all isolates of the targeted gene have high similarity (98.9%) with France isolate (KP994842) (Fig. 6).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	
1		39.1	100.0	40.7	100.0	86.6	40.7	41.1	40.7	40.7	42.3	1
2	129.9		28.6	97.4	28.6	29.2	97.1	97.1	96.5	97.1	30.3	2
3	0.0	129.9		37.3	58.3	86.4	37.3	37.6	37.3	37.3	28.1	3
4	124.8	2.4	141.9		6.3	9.8	98.8	98.8	98.4	98.9	6.9	4
5	0.0	130.4	1.6	124.5		84.9	40.7	41.1	40.7	40.7	41.9	5
6	9.8	126.4	8.1	129.3	11.5		38.6	38.6	38.4	38.6	28.0	6
7	123.3	2.4	140.9	1.1	123.1	128.5		98.9	99.3	99.6	6.3	7
8	121.4	2.4	139.5	1.1	121.2	128.5	0.8		99.2	99.0	6.3	8
9	123.3	3.0	140.9	1.3	123.1	129.8	0.3	0.7		99.6	6.3	9
10	123.3	2.4	140.9	1.0	123.1	128.5	0.2	0.8	0.4		6.3	10
11	107.6	186.5	106.2	201.2	109.4	114.0	217.2	217.2	217.2	217.2		11
	1	2	3	4	5	6	7	8	9	10	11	

1 MK256950 A. gervaisi
2 MG879372 South India
3 MG888733 Kerala, India
4 MF102097 USA
5 MK256949 A. varanense
6 MH282856 Amblyomma sp
7 KJ584369 A. rotundatum.
8 FJ464422 A. parvum.
9 L76344 A. maculatum
10 L76345 A. tuberculatum
11 MH047859 Demodex canis

Figure 5: Distance matrix of different sequences of *Amblyomma gervaisi and A. varanense* 18S rRNA gene sequences available in GenBank

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		100.0	71.3	69.1	68.9	68.9	69.6	69.1	67.2	71.3	69.6	68.9	67.2	69.6	69.6	1	VPATickCb 1
2	0.0		71.3	69.1	68.9	68.9	69.6	69.1	67.2	71.3	69.6	68.9	67.2	69.6	69.6	2	VPATickCb 2
3	2.3	2.3		95.5	95.9	95.9	95.4	95.5	90.5	100.0	95.4	95.9	90.5	95.4	95.4	3	JQ480818
4	1.8	1.8	0.9		99.6	99.6	99.3	100.0	94.3	99.1	99.3	99.6	94.3	99.3	99.3	4	KP994829
5	2.0	2.0	0.5	0.4		100.0	99.1	99.6	94.1	99.5	99.1	100.0	94.1	99.1	99.1	5	KP994840
6	2.0	2.0	0.5	0.4	0.0		99.1	99.6	94.1	99.5	99.1	100.0	94.1	99.1	99.1	6	KY026064
7	1.1	1.1	1.1	0.7	0.9	0.9		99.3	95.0	98.9	100.0	99.1	95.0	100.0	100.0	7	KP994827
8	1.8	1.8	0.9	0.0	0.4	0.4	0.7		94.3	99.1	99.3	99.6	94.3	99.3	99.3	8	KP994830
9	0.6	0.6	2.3	1.9	2.1	2.1	1.1	1.9		97.8	98.9	98.0	100.0	98.9	98.9	9	KP994841
10	2.3	2.3	0.0	0.9	0.5	0.5	1.1	0.9	2.3		95.4	95.9	90.5	95.4	95.4	10	JQ480818
11	1.1	1.1	1.1	0.7	0.9	0.9	0.0	0.7	1.1	1.1		99.1	95.0	100.0	100.0	11	KP994828
12	2.0	2.0	0.5	0.4	0.0	0.0	0.9	0.4	2.1	0.5	0.9		94.1	99.1	99.1	12	KP994839
13	0.6	0.6	2.3	1.9	2.1	2.1	1.1	1.9	0.0	2.3	1.1	2.1		98.9	98.9	13	KP994842
14	1.1	1.1	1.1	0.7	0.9	0.9	0.0	0.7	1.1	1.1	0.0	0.9	1.1		100.0	14	KP994828
15	1.1	1.1	1.1	0.7	0.9	0.9	0.0	0.7	1.1	1.1	0.0	0.9	1.1	0.0		15	KP994827
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Fig. 6: Distance matrix of different closely related sequences of *Coxiella burnetii* 16S rRNA gene sequences available in GenBank

3.5 Phylogenetic relationship

The phylogenetic tree based on maximum likelihood method with Tamura-Nei model for distance calculation based on 253bp region of 18SrRNAgene is shown in Figure 7 and for *C. burnetii* in Figure 8. Isolates from Kerala, South India (MG888733), USA (MH282856) and the newly generated sequences formed separate clade in the neighborhood joining tree with bootstrap value (98%).Isolates from France (KP994842) formed separate clade in the neighbour joining tree with high bootstrap support (91%).

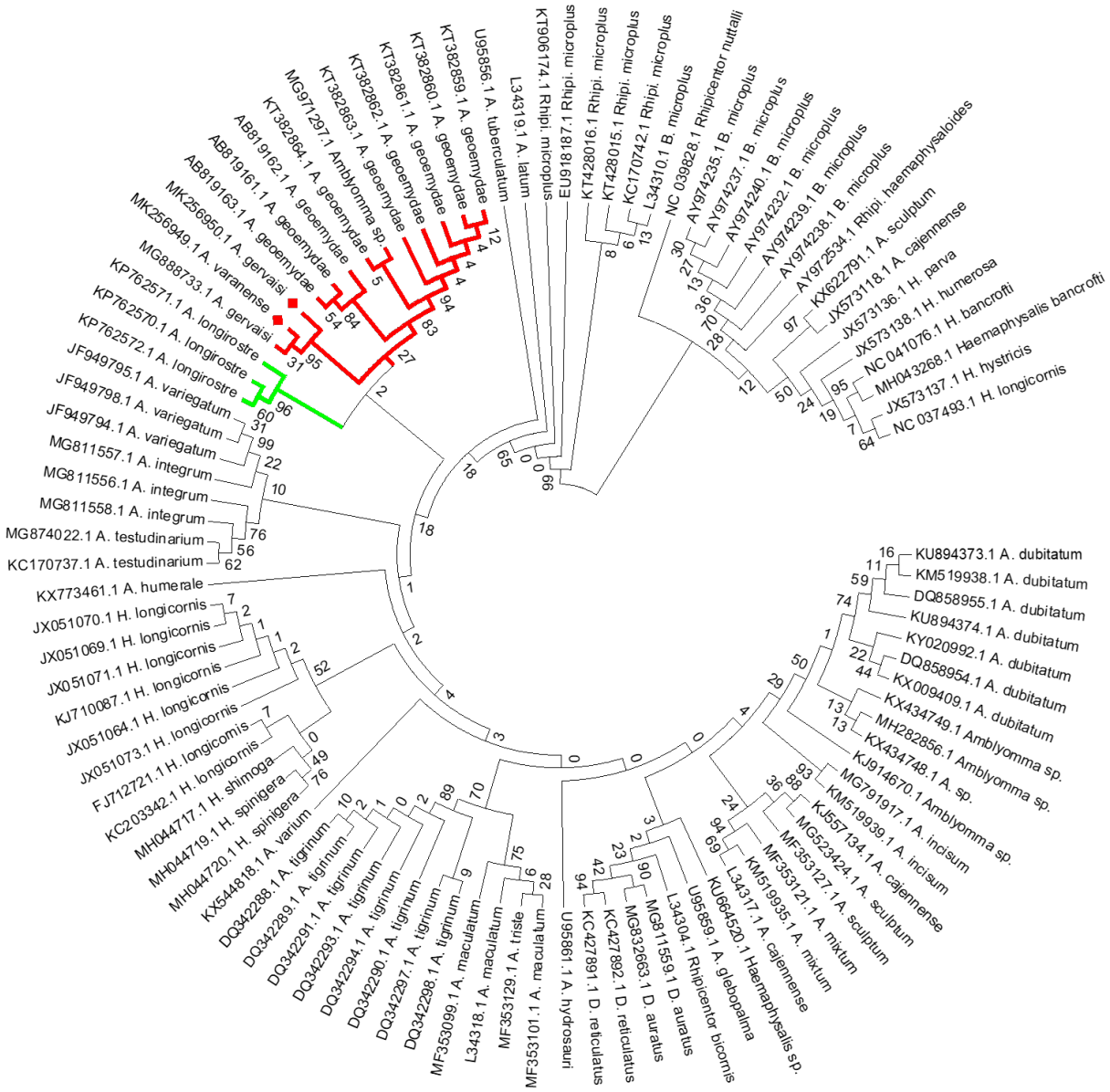

Figure 7: Phylogenetic analysis of *Amblyomma varanense* and *A. Gervaisi* based on maximum likelihood tree (1000 bootstraps) constructed with 18S rRNA sequences of various origins. Published GenBank accession numbers are noted for each species. Bootstrap values are indicated on branches. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 100 nucleotide sequences. There were a total of 140 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

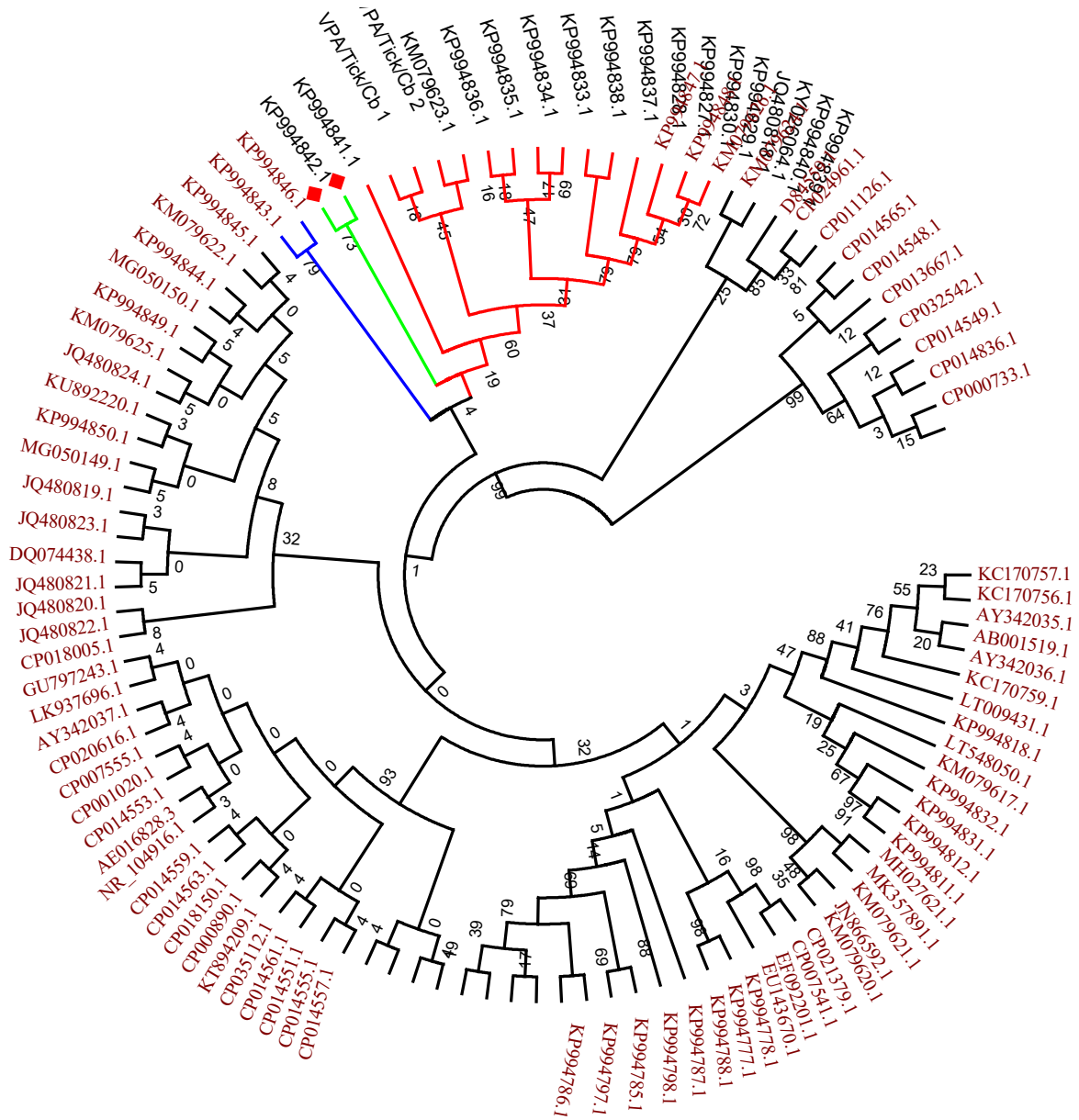

Figure 8: Phylogenetic analysis of *Coxiella burnetii* based on maximum likelihood tree (1000 bootstraps) constructed with 16S rRNA sequences of various origins. Published GenBank accession numbers are noted for each species. Bootstrap values are indicated on branches. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 102 nucleotide sequences. There were a total of 522 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

4. Discussion

Ticks act as a link for different disease-causing agents between animal reservoirs and human hosts. The aim of this study was to characterize both morphologically and molecularly two most commonly occurring ticks *i.e. A. gervaisi* and *A. varanense* in snakes and to identify endosymbiotic relationship if any between these two species of ticks. Present study reported that the prevalence of tick varied between different species of snakes at least in north eastern region of India. Our study displayed higher tick burdens in female snakes than conspecific males. This sex-biased parasitism may be due to differences in immunocompetence, costs of reproduction or from behavioral and/or niche divergence [25]. Most of the recovered ticks were adult. This information suggests that larvae and nymphs of both species may utilize different hosts during their life cycle. The higher tick burden was reported in cobra in contrast with other three species of snakes which is broadly comparablewith previous works [26, 27] but lower than in some other reptiles (*e.g.* > 98% in *Varanus komodensis*) [28]. Our study also noticed seasonal disparity of tick burden on snakes which may be due to more frequent movement of tropical snakes during wet season consequently increase the chance of contact with ticks [29]. Catherine *et al*. [30] reported the infestation of *Am. gervaisi* on reticulated python (*Python reticulatus*), Indian Rock python (*Py. molurus*), Spectacled cobra (*N. naja*) and Rat snake (*Pt. mucosa*) from captive snakes at Chennai snake park Trust (Guindy), Arignar Anna Zoological Park (Vandalur) and Rescue Centre (Velachery) in Tamil Nadu.

The SEM observation of both species of ticks is discussed along with light microscopy. Basis capitulum was roughly flask shaped in *A.gervaisi* but it was rectangular in *A. varanense.* A similar observation was also reported by Ghosh et al. [31] in *A. helvolum* which also showed flask shaped basis capitulum like *A.gervaisi*. The most distinguishing feature observed in *A.varanense* was the sub ventral position of the mouthparts in contrast to other hard ticks. The dental formula on the hypostome of *A.gervaisi* was 3/3 whereas in *A.varanense* it was 4/4. The structure of mouth palps was more or less similar in both species, although palp head was less hairy in *A.varanense* than *A.gervaisi.* Numerous punctuations were uniformly distributed throughout the scutal surface of both *A.varanense* and *A.gervaisi.* The male genital, the anus, hallers' organs and spiracles looked almost similar both under light microscopy and SEM. Ghosh *et al*. [32] reported the occurrence of snake associated ticks, *Am. gervaisi* and *Am. helvolum* from both natural and captive habitats. They described the sexual dimorphism of *Am. gervaisi* and *Am.*

helvolum by using SEM observations and made a comparison with other available SEM studies on ixodid tick species. Ghosh *et al.* [33] also elaborated on the detail anatomy of Haller's organ of *Am. gervaisi* and *Am. helvolum* by using SEM and TEM.

The tegument plays an important role in the parasites' homeostasis such as absorption and exchange of nutritive and waste molecules and ionic regulation between the interior of the parasites and the surrounding host fluid. The biological success of arthropod is directly related with its integument structure, because of its locomotive, protective and support properties [26]. As in other arthropods, the integument of *A. gervaisi* and *A. varanense* composed of a cuticle and an epidermal cell layer. The cuticle was clearly divided into an outer epicuticle and an inner procuticle. The ultra structure of the cuticle of both species of ticks in the present study was similar to that observed in *Hyalomma anatolicum anatolicum* [33], *Ixodes ricinus* [34] and *Rhipicephalus sanguineus* [35].

This study genetically identified *A.gervaisi* and *A.varanense* based on the 18S rRNA gene of both species collected from four species of snakes from different NE regions of India. Although species determination and differentiation of *Amblyomma* are traditionally based on morphological features of the adult ticks, a DNA based approach provides the guide lines for investigation of the genetic variation at the individual base pair level and gives much more direct pathway for genetic diversity between and within species of *Amblyomma* ticks [36]. In the present study, sequence analysis of the 18S rRNA gene has been used to compare the phylogenetic relationship of *A. gervaisi* and *A. varanense* as well as other *Amblyomma* species. The result suggests that both species form a cluster in the same clade whereas other *Amblyomma* species form a separate clade with sequence diversity ranges from 39.1% to 100%.

The presence of *C. burnetii* in both species of ticks infesting various species of snakes highlighted the possible importance of these ticks in the epidemiology of *C. burnetii* in India. The overall prevalence of *C. burnetii* in our present study, a relevant prevalent rate (10%) was observed in ticks infesting snakes which were comparable to the finding of Hornoket al. [37] who also recorded the overall prevalence of *C. burnetii* (10.8%) in ticks infesting cattle. Many workers reported *C. burnetii* from various animals suggesting the zoonotic potential of them as reservoir host for Q fever. So, ticks can be contaminated while feeding on infected snakes. Considering to the fact that *A. gervaisi* and *A.varanense* arethe predominated species of ticks

infesting snakes in India, there is every chance of transmission of *C. burneti* from snakes to humans in this study area. Our phylogenetic analyses indicate that the *Coxiella* symbiont of *Amblyomma* is unique, but is related to *Coxiella* symbionts ofother hard ticks. These symbionts group together within a larger monophyletic group of *Coxiella* symbionts of both soft and hard ticks. Because the phylogenetic relatedness of all knowntick-associated *Coxiella* symbionts tend to be congruent to the phylogenetic relatedness of their specific tick hosts and because ticks generally have very high infection rates (e.g., 100%), this finding suggests that the original infection occurred in anancestor common to the 2 main tick families, Argasidae (soft ticks)and Ixodidae (hard ticks). However, further testing of more tickspecies and populations is needed to firmly establish this conclusion. Previous studies with insects and symbionts have reportedcongruent phylogenies between hosts and their bacterial symbionts [38, 39]. On the other hand,our analyses also suggest that at a few points of the tick-*Coxiella* coevolution, the bacterium evolved to pathogenic agents, resultingin the organism's *C. burnetii* and Candidatus '*Coxiella avium'*. While the former pathogen is the etiological agent of Q fever worldwide [40], the latter agent was recently described causing severe disease in birds [41].

From medical point of view, ticks are important vectors as well as reservoir host of diseases in human beings. The importance of the present work is of two fold. Firstly, it provides basic information on the occurrence of snake ticks caught in wild [42] that would help in the quarantine process during trading of snakes. Secondly, it shows that snake ticks have low species specificity that would increase the disease contamination among the snakes and to develop awareness to the authorities of Zoo garden and Snake Park for management of the snakes caught from the wild. Wild caught snakes frequently have ticks. There are only two ways the snake could pick up ticks – either through the wild or through direct contact with another snake that already has ticks. Increase in international wildlife trade, especially reptiles [43], will cause high risks of introduction of exotic ticks along with the pathogens in new geographic areas [44].

In conclusion, this study describes the detail morphology of *A.gervaisi* and *A.varanense* both under light microscopy and electron microscopy and also provides the genetic identification of the 18SrRNA gene of both ticks collected from different species of snakes. Because of the likely long co-evolution process and high infection rates in tick populations, it is possible that the infection by *Coxiella* symbionts is beneficial for ticks. Further studies employingmicroscopic and biochemical methods are needed to confirmthe symbiotic nature of the *Coxiella* agent of

Amblyomma and to testa possible mutual benefit of this tick-bacterium interaction.The study also detected *C. burneti* from both species.Future study focusing on feeding habitat and vector capacity of these two distinctive species may give valuable information about life cycle and epidemiological features of tick borne pathogens transmitted by *A. gervaisi* and *A. varanense* to animals and humans.

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