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Isolation and Genetic Characterization of *Cryptosporidium* from Captive Wildlife of India

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ABSTRACT

Background: Cryptosporidiosis is an emerging zoonotic protozoan disease caused by *Cryptosporidium* spp. The infection was reported worldwide from domestic animals and humans, including wild animals. From India, no such reports were published on *Cryptosporidium* infection in captive wildlife. Hence, a pilot study was conducted to report the occurrence of *Cryptosporidium* infection in captive wildlife of India.

Methods: Faecal samples (n=788) were collected from 127 captive wildlife species of three zoological parks *viz*., Sri Venkateswara Zoological Park (SVZP), Tirupati (n=242); Indira Gandhi Zoological Park (IGZP), Visakhapatnam (n=218); Nehru Zoological Park (NZP), Hyderabad (n=328) and screened for *Cryptosporidium* infection. Preliminary screening of faecal samples was done for the detection of *Cryptosporidium* oocysts by modified Ziehl-Neelsen (mZN) staining method and the test positives were confirmed by nested PCR targeting 18S rRNA gene. Nested PCR amplicons were sequenced for determining the *Cryptosporidium* species. The resultant data were statistically analyzed by Fisher/Chi square, fisher exact test using SPSS software v 17.0.

Result: In mZN staining method, 7.23 per cent of isolates were found to be positive for *Cryptosporidium* and the highest rate of infection was detected in wildlife at NZP, Hyderabad (8.23%), followed by SVZP, Tirupati (7.44%) and IGZP, Visakhapatnam (5.50%). *Cryptosporidium* positive faecal samples by mZN staining were further confirmed by nested PCR and positive amplicons were sequenced for determination of *Cryptosporidium* species. Genetic characterization revealed five species *viz*., *Cryptosporidium parvum; C. ryanae, C. suis*, *C. muris* and *Cryptosporidium* avian genotype III. The study conclude that, *Cryptosporidium* infection was prevalent in the captive wildlife from the zoological parks of India and species variation was marked among the wildlife. Based on the available literature, the current study is the first of its kind on the prevalence of *Cryptosporidium* in captive wildlife from India.

Key words: *Cryptosporidium,* Captive wildlife, mZN staining, Nested PCR.

INTRODUCTION

Nature's beautiful creatures are the wildlife and are part of our bio-diversity. Sometimes, these wild animals may also act as carriers for several zoonotic parasites to domestic animals and humans. Introduction of faecal pathogens from an infected host to the environment, adversely leads to a potential source for the susceptible hosts. Among these zoonotic enteric parasites, *Cryptosporidium* was such a pathogen can lead both wildlife and human population to a situation involving exposure to danger (Appelbee *et al.,* 2005).

The genus *Cryptosporidium* is classified under the phylum: Apicomplexa, class: Sporozoasida, sub-class: Coccidiasina, order: Eucoccidiorida, sub-order: Eimeriorina, family: Cryptosporidiidae (Fayer and Ungar, 1986). Currently, 39 *Cryptosporidium* species/genotypes have been recognized as valid and half of them were identified in mammals (Firoozi *et al.,* 2019). Of these, *Cryptosporidium parvum* is a public health significant pathogen recognized throughout the world.

Cryptosporidium is an obligate protozoan parasite, which infects microvillous border of the gastrointestinal tract and causes cryptosporidiosis in a wide range of hosts. Viable *Cryptosporidium* oocysts are transmitted through the faeco-oral route and are shed in the excreta of infected hosts (Fayer *et al.,* 1997). *Cryptosporidium* is becoming as a significant cause of moderate to severe diarrhoea in developing nations and attracted attentiveness due to several intense human outbreaks (MacKenzie *et al*., 1994).

Through wild animals, *Cryptosporidium* could be a serious public health problem due to oocyst contamination in the soil and water (Fayer and Xiao, 2008). Numerous reports on *Cryptosporidium* species/genotypes of wild animals have been documented worldwide (Lim *et al*., 2007; Ludwig and Marques, 2011; Samra *et al.,* 2011). In India, the great majority of cryptosporidiosis infection was reported in domestic animals of economic importance *viz*., cattle (Khan *et al.,* 2010; Venu *et al.,* 2011; Bhat *et al.,* 2013), sheep (Ahamed *et al.,* 2013; Maurya *et al.,* 2013) and goat

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(Maurya *et al.,* 2013; Rakesh *et al.,* 2014). But, information on wildlife cryptosporidiosis is not available. Therefore, a pilot study was undertaken with an objective aimed to detect *Cryptosporidium* infection in captive wildlife of India.

MATERIALS AND METHODS

Study area

Sri Venkateswara Zoological Park (SVZP), Tirupati lies at 13.65°N and 79.42°E in Chittoor district of south Indian state, Andhra Pradesh and it is one of the largest zoo parks in Asia and named as a 'house of wild birds'. Indira Gandhi Zoological Park (IGZP), Visakhapatnam of Andhra Pradesh state located between 17.7041°N latitude and 83.2977°E longitude. The zoo park area is surrounded by Eastern Ghats and Bay of Bengal. Nehru Zoological Park (NZP), Hyderabad is located in Telangana state at 17.366°N latitude and 78.476°E longitude in the northern part of the Deccan Plateau.

Sample collection

Faecal samples were collected from apparently healthy captive wildlife of three zoological parks *viz*., SVZP, Tirupati; IGZP, Visakhapatnam and NZP, Hyderabad during 2013-14. Prior permission was obtained for the collection of faecal samples from captive wildlife (Rc. No. 39170/2013/WL-3 dated 20-11-2013 of Principal Chief Conservator of Forests (WL) and Chief Wildlife Warden, Forest Department, Government of Andhra Pradesh, Hyderabad). Seven hundred and eighty eight fresh faecal samples were collected, immediately after defaecation (Table 1). With the help of zoo attendants, samples were collected only once from individual enclosures/cages by wearing a disposable hand glove and transferred into a 50ml sterile plastic screw capped sample container. The samples were separately labeled and are transported on ice to the laboratory and stored at 4°C till further processing.

Screening of samples

The collected samples were screened at College of Veterinary Science, Tirupati. Faecal suspension was prepared by mixing half gram of faecal sample with 1.4 ml of distilled water in a 2 ml microcentrifuge and vortexed thoroughly till a homogenous suspension was obtained. Unconcentrated faecal smears were prepared, air dried and methanol fixed smears were subsequently stained by mZN staining method (Henricksen and Pohlenz 1981). A minimum of 300 microscopic fields of the stained smear was first observed under a compound microscope at a magnification of 400X and then 1000X. Samples were considered as positive even when a single oocyst was observed.

DNA extraction

Genomic DNA was extracted from mZN test positive faecal samples using QIAamp® Fast DNA stool mini kit (Qiagen, Germany). The eluted DNA was tested for its purity with Nanodrop® and DNA samples were considered to be of sufficient purity if the absorbance ratio was 1.7 and above. The DNA samples were stored at -20°C for later use.

Nested PCR

A two-step nested PCR protocol was followed to amplify ~830 bp fragment of the 18S rRNA gene of *Cryptosporidium* (Xiao *et al*. 1999; Xiao *et al*. 2001). The primers used in the study were custom synthesized at Eurofins Genomics Ltd. (MWG), Bangalore. The external (Forward: 5′-TTC TAG AGC TAA TAC ATG CG-′ and Reverse: 5′- CCC ATT TCC TTC GAA ACA GGA-3′) and internal (Forward: 5′- GGA AGG GTT GTA TTT ATT AGA TAA AG-3′ and Reverse: 5′- AAG GAG TAA GGA ACA ACC TCC A-3′) primers were used at a concentration of 10 pmol/µl. Twenty five microlitres of PCR mixture was prepared for primary PCR (forward and reverse primers - 1.0 µl each; PCR master mix (2X) - 12.5 µl; DNA template 2.0 µl and nuclease free water 8.5 µl). The primary PCR cycling conditions include with an initial melting temperature at 94°C for 3 min. followed by denaturation at 94°C for 45 sec. annealing temperature at 59°C for 45 sec. and an extension for one minute at 72°C for 35 cycles with a final extension at 72°C for 7 min. Secondary PCR was performed using internal primers and the reaction volume and composition similar to primary PCR, except using DNA template from primary PCR amplicon. The cycling conditions for secondary PCR was similar to primary PCR with a slight variation in denaturation time *i.e*., for 30 sec. and annealing temperature at 58°C for 90 sec. and extension time was allowed for 2 min.

Sequencing

A representative nested PCR amplicons were subjected to bi-directional sequencing to determine *Cryptosporidium* species. Cycle sequencing of 18S rRNA *Cryptosporidium* gene was carried out in an automated DNA sequencing facility at Eurofins Genomics Ltd. (MWG), Bangalore. Each sequence was compared by BLAST with the respective *Cryptosporidium* species reference sequences retrieved from GenBank and the generated sequences were submitted in the GenBank.

Table 1: Particulars of faecal sample collection.

Zoo park	Kind of wildlife						Total
	Herbivores	Carnivores	Primates	Birds	Rodents	Reptiles	
SVZP, Tirupati	72	72	14	72	6	6	242
IGZP, Visakhapatnam	85	70	11	44	3	5	218
NZP, Hyderabad	123	53	20	115	2	15	328
Total	280	195	45	231	11	26	788

Phylogenetic analysis

Phylogenetic tree was constructed with sequences generated in this study and reference sequences obtained from GenBank. Sequences were aligned following ClustalW algorithm included in the Megalign module (DNASTAR Inc., Madison, WI). Phylogenetic inference was performed by the neighbor-joining method as implemented in the MEGA4 program (Tamura *et al*. 2007). *Plasmodium falciparum* was used as an out group to root the neighbor-joining tree since the construction of an unrooted tree showed it to be the most divergent member under analysis. The branch reliability was assessed by the bootstrap method with 1000 replications.

Statistical analysis

The results of the study have been analyzed by Fisher/Chi square, Fisher Exact test using SPSS software v 17.0; Pairwise comparisons were carried out using Fishers test with Bonferroni correction.

RESULTS AND DISCUSSION

In mZN staining, fifty seven isolates (7.23%) out of 788 faecal samples screened were found to be positive for *Cryptosporidium* oocysts (Fig 1). The highest prevalence of *Cryptosporidium* infection was detected in the wildlife at NZP, Hyderabad (8.23%) followed by SVZP, Tirupati (7.44%) and IGZP, Visakhapatnam (5.50%). The highest rate of *Cryptosporidium* infection was observed in rodents (18.18%) followed by reptiles (11.54%), primates (11.11%), herbivores (9.29%), birds (7.79%) and the lowest was noticed in carnivores (1.54%). Statistical analysis revealed that, no significant ($p<0.05$) association was observed between the location of the zoological parks and the prevalence of *Cryptosporidium* infection (Table 2).

Sri Venkateswara Zoological Park (SVZP), Tirupati

Cryptosporidium infected wildlife in SVZP are eight herbivores, one primate and nine birds. In herbivores, one elephant (*Elephas maximus*), two sambar deer (*Cervus unicolor*), two swamp deer (*Cervus duvaceli*) and three spotted deer (*Axis axis*) were found to be infected. Stump tail macaque (*Macaca artoides*) is the only primate infected. In birds, three ring necked parakeets (*Psittacula krameri*), two emu (*Dromaius novaebollandiae*) and one each of

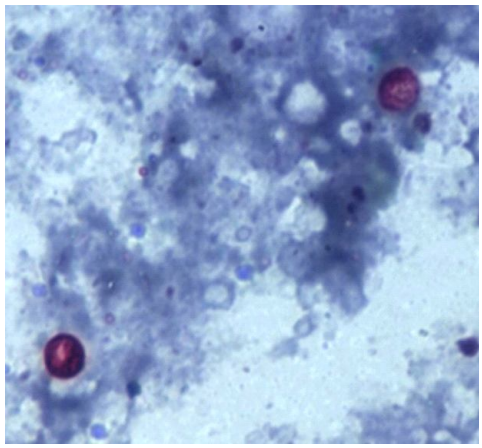


Fig 1: *Cryptosporidium* oocysts (mZN stained pink bodies, 1000x).

Table 2: Details of *Cryptosporidium* positives in wildlife of three zoological parks by mZN staining method.

Wildlife	Zoological park			Total	#Fisher/ Chi square p-value
	SVZP, Tirupati	IGZP, Visakhapatnam	NZP, Hyderabad		
Herbivores	8/72 (11.11%)	7/85 (8.24%)	11/123 (8.94%)	26/280 (9.29%)	0.813 NS
	[4.9-20.7]^{b}	[3.4-16.2]^{a}	[4.5-15.4]^{b}	[6.2-13.3]	(x^2=4.113; df=2)
Carnivores	0/72 (0.0%)	1/70 (1.43%)	2/53 (3.77%)	3/195 (1.54%)	
	[0.0-0.0]^{a}	[0.04-7.7]^{a}	[0.46-13.0]^{a}	[0.32-4.4]	0.193 NS
Primates	1/14 (7.14%)	1/11 (9.09%)	3/20 (15.0%)	5/45 (11.11%)	
	[0.18-33.9]^{ab}	[0.23-41.3]^{ab}	[3.2-37.9]^{b}	[3.7-24.1]	0.844 NS
Birds	9/72 (12.5%)	1/44 (2.27%)	8/115 (6.96%)	18/231 (7.79%)	
	[5.9-22.4]^{ab}	[0.06-12.0]^{a}	[3.1-13.2]^{b}	[4.7-12.0]	0.122 NS
Rodents	0/6 (0.0%)	2/3 (66.67%)	0/2 (0.0%)	2/11 (18.18%)	
	[0.0-0.0]^{ab}	[9.4-99.2]^{b}	[0.0-0.0]^{ab}	[2.3-51.8]	0.073 NS
Reptiles	0/6 (0.0%)	0/5 (0.0%)	3/15 (20.0%)	3/26 (11.54%)	
	[0.0-0.0]^{ab}	[0.0-0.0]^{ab}	[4.3-48.1]^{b}	[2.4-30.2]	0.556 NS
Total	18/242 (7.44%)	12/218 (5.50%)	27/328 (8.23%)	57/788 (7.23%)	
	[4.5-11.5]	[2.9-9.4]	[5.5-11.8]	[5.5-9.3]	
*Fisher exact	12.189	14.480	6.173		
Sig	0.019*	0.007**	0.254 NS		

Note: Figures shown as number positive/number examined; Values in ( ) indicate percent positives; Values in [ ] represents the lower and upper 95% confidence limits; NS: Non significant; *Fisher/Chi square for wildlife species within a zoological park; # Fisher/Chi square for zoological parks within a species; Fisher exact test using SPSS software v 17.0; Pairwise comparisons were carried out using Fishers test with Bonferroni correction; Different superscripts are significantly different ($p<0.05$).

Alexander parakeet (*Psittacus eupatria*), common peacock (*Pavo cristatus*), parrot (*Eclectus roratus*) and Fisher's love bird (*Agapornis fischeri*) was infected. None of the carnivore, rodent or reptile faecal samples were found positive (Table 2).

Indira Gandhi Zoological Park (IGZP), Visakhapatnam

Cryptosporidium positives of IGZP include seven herbivore samples, in which three of barking deer and the remaining was sambar deer. One carnivore (palm civet), a primate (marmoset), a wild bird (common peacock) and two rodents (giant squirrels) were found to be positive. Except reptile faecal samples, other species of wildlife were found positive for *Cryptosporidium* (Table 2).

Nehru Zoological Park (NZP), Hyderabad

Among the *Cryptosporidium* positive samples, eleven were herbivores followed by eight birds, three primates, three reptiles, two carnivores and none in the rodent samples. In herbivores, one elephant (*Elephas maximus*), five spotted deer (*Axis axis*), three barking deer (*Muntiacus muntjak*) and two sambar deer (*Cervus unicolor*) samples were infected. In carnivores, one lion (*Panther leo*) and a hyena (*Crocuta crocuta*) sample; whereas in primates, two sacred baboons (*Papio hamadryas*) and one Nilgiri langur (*Presbytis johni*) were infected. One king cobra (*Ophiophagus hanna*) and two green iguana (*Iguana iguana*) were the reptiles among the infected. In wild birds, two common peacocks (*Pavo cristatus*) and one each grey cockatiel (*Nymphicus hollandicus*), khaliz pheasant (*Lophura leucomelanos*), ostrich (*Struthio camelus*), ring necked parakeet (*Psittacula krameri*), silver pheasant (*Lophura nucthemera*) and a white peacock (*Pavo cristatus*) were infected (Table 2).

In the current study, the prevalence of *Cryptosporidium* infection ranged between 5.5-8.23% among the three zoological parks. In contrast to the present findings, a low prevalence was reported by several authors in different kinds of wildlife (Lim *et al.,* 2007; Majewska *et al.,* 2009; Bernardi *et al.,* 2014). Good level of hygiene and management prevailed in the wildlife habitations may have accounted for the low prevalence of infection. Several researchers reported a high prevalence of *Cryptosporidium* in various wildlife faecal samples (Sturdee *et al.,* 1999; Ekanayake *et al.,* 2006; Samra *et al.,* 2011; Radhy *et al.,* 2013). Environmental contamination, high output of oocysts, chronic infection, young age, low immunity, poor hygiene/management could lead to the persistence of *Cryptosporidium* and contribute to the high rate of infection. The prevalence rate of *Cryptosporidium* in the current study was comparable to other observations (Karasawa *et al.,* 2002; Gonzalez-Moreno *et al.,* 2013; Diakou *et al.,* 2015). The reason for the similarity may be due to the similar management conditions, including the detection methods and design of the study.

The present findings demonstrated that, among the *Cryptosporidium* positives, the highest infection (47.37%) was recorded in NZP, Hyderabad, followed by SVZP, Tirupati (31.58%) and IGZP, Visakhapatnam (21.10%). Other studies have shown that there could be substantial differences in the prevalence rate, which might be influenced by a range of factors, including analytical methods, geographical differences, age, sex, composition of sampled animals, sample size, sampling season and intermittent oocyst shedding (Hamnes *et al.,* 2006; Castro-Hermida *et al.,* 2011). Further, the high prevalence in wildlife could be attributed to their shared habitat (Hope *et al.,* 2004) and the possibility of co-incidental sampling of several faecal samples from the same individual (Ravaszova *et al.,* 2012). With or without prior concentration of faecal smears may be one of the reasons to influence on lower or higher prevalence rates of cryptosporidiosis in wildlife (Garcia *et al.,* 1983).

In the current study, genomic DNA was extracted from mZN staining positive faecal samples (n=57). The DNA concentration range was 10.6 to 79.6 nanograms/microlitre and the purity range was estimated at 1.7 to 2.88. The isolated DNA samples were subjected to nested PCR targeting 18S rRNA gene for detection of *Cryptosporidium* and were successful by visualizing the expected amplicons (~830 bp) in gel electrophoresis (Fig 2). Representative positive amplicons by nested PCR were subjected to bi-directional sequencing to determine *Cryptosporidium* species. The sequences obtained were confirmed by BLAST analysis and five *Cryptosporidium* species were identified

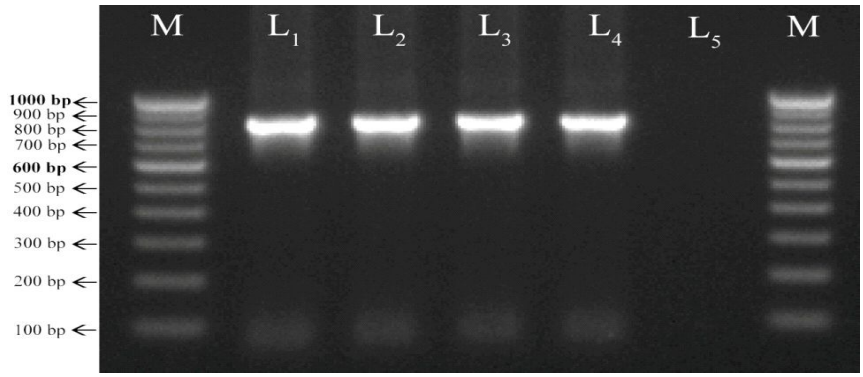


Fig 2: Agarose gel electrophoresis of nPCR amplified products of 18S rRNA. gene of *Cryptosporidium* from wildlife faecal samples. (M: 100bp DNA ladder; L1 - L4: nPCR amplicons of positive isolates; L5: Non template control).

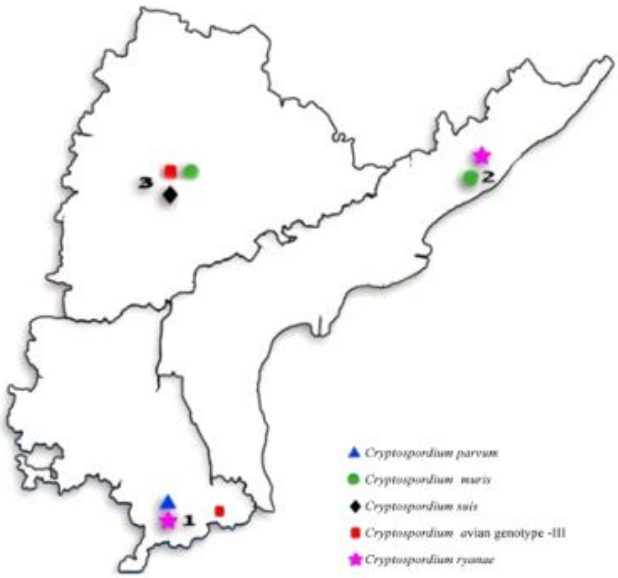
based on the per cent nucleotide identity (98-99%) with the respective sequences from GenBank. The genotyped *Cryptosporidium* were *C. parvum; C. ryanae, C. suis*, *C. muris* and *Cryptosporidium* avian genotype III (Table 3). The nucleotide sequences generated in the current study are available in GenBank (KX668207, KX668208, KX668209, KX668210, KX668211, KX668212 and KX668213). Phylogenetic tree was constructed with the respective GenBank retrieved reference sequences. The isolates of *C. parvum*, *C. suis*, *C. ryanae*, C. avian genotype III and *C. muris* was grouped into separate clades with respective reference sequences (Fig 4).

In the present study, *Cryptosporidium* species were varied among the captive wildlife of three zoological parks. Zoonotic species, *C. parvum* was identified in herbivore isolate of SVZP, Tirupati. Various authors reported *C. parvum* in wildlife from worldwide (Xiao *et al.,* 2000; Silva *et al.,* 2003; Xiao *et al.,* 2004; Lv *et al.,* 2009; Nakamura *et al.,* 2009).

The samples of sambar deer at SVZP, Tirupati and IGZP, Visakhapatnam were identified as *C. ryanae*. This observation suggest that, an increased interaction between the wildlife and domestic animals playing an important role as a source of infection (Venu *et al.*, 2012). Further, the sampled zoological parks were in the vicinity of the urban environment, so that every possibility to spread the infection between the domestic animals and wildlife. However, further investigations are required to ascertain this kind of likelihood

Table 3: *Cryptosporidium* species of wildlife of three zoological parks of South India.

Zoological park	Isolate	Wildlife	*Cryptosporidium* spp.
VZP, Tirupati	SV251	Elephant (*Elephas maximus*)	*C. parvum*
	SV125	Sambar deer (*Cervus unicolor*)	*C. ryanae*
	SV232	Ring necked parakeet	*Cryptosporidium* avian genotype III
	SV234	Ring necked parakeet	*Cryptosporidium* avian genotype III
IGZP, Visakhapatnam	IG932	Sambar deer (*Cervus unicolor*)	*C. ryanae*
	IG935	Sambar deer (*Cervus unicolor*)	*C. ryanae*
	IG898	Giant squirrel	*C. muris*
	IG899	Giant squirrel	*C. muris*
NZP, Hyderabad	N328	Sambar deer (*Cervus unicolor*)	*C. suis*
	N330	Sambar deer (*Cervus unicolor*)	*C. suis*
	N422	Grey cockatiel (*Nymphicus hollandicus*)	*Cryptosporidium* avian genotype III
	N594	Nilgiri langur (*Presbytis johnii*)	*C. muris*



1. SRI VENKATESWARA ZOOLOGICAL PARK, TIRUPATI
2. INDIRA GANDHI ZOOLOGICAL PARK, VISAKHAPATNAM
3. NEHRU ZOOLOGICAL PARK, HYDERABAD.

Fig 3: Distribution of *Cryptosporidium* species in captive wildlife of South India.

and mode of transmission. The present finding corroborate with the earlier observation by Garcia-Presedo *et al.* (2013b). In NZP, Hyderabad, *Cryptosporidium* positive isolates of sambar deer have 98% identity with *Cryptosporidium suis.* Similar kind of results could not be traced out in the literature for comparison; however *C. suis* was reported in wild boars (Garcia-Presedo *et al.,* 2013ba).

Cryptosporidium muris was identified in two giant squirrels from IGZP, Visakhapatnam and in one Nilgiri langur isolate of NZP, Hyderabad in the present investigation. Comparable findings were observed in wild rodents (Sturdee *et al.,* 1999); Eastern grey squirrels (Feng *et al.,* 2007) and wild, laboratory and pet rodents (Lv *et al.,* 2009). In contrast, *C. parvum* was observed in captive lemurs and other *Cryptosporidium* species in primates by Silva *et al.,* (2003) and Ekanayake *et al.,* (2006).

In the present study, *Cryptosporidium* avian genotype III was identified from ring necked parakeet samples of SVZP, Tirupati and in grey cockatiel bird isolate of NZP, Hyderabad (Table 3; Fig 3). Our findings were in agreement with most observations in wild birds located in various countries (Ng *et al.,* 2006; Romulo *et al.,* 2008; Nakamura *et al.,* 2009; Wang *et al.,* 2011).

In the present investigation, only three *Cryptosporidium* positives out of 195 carnivore faecal samples were noticed. The finding could be justified with their stay in the individual enclosures/cages for longer periods, particularly lions and tigers, concrete floors and regular cleaning may be the reasons for chance of low infection. The origin of animals in the zoo/quarantine systems followed, regular prophylactic treatment, supply of good quality of water and balanced di*et al*so contribute to the low prevalence of infection (Matsubayashi *et al.,* 2005). Several factors may be responsible for the differences in distribution of *Cryptosporidium* species in wildlife from one study to another could be explained by variations in wild animal species, nursing conditions of the host, season of the sample collection as well as the sanitary conditions inside and around the zoological parks. Some of these factors may act individually or collectively to increase the risk factors associated with transmission and prevalence of *Cryptosporidium* in wild animals.

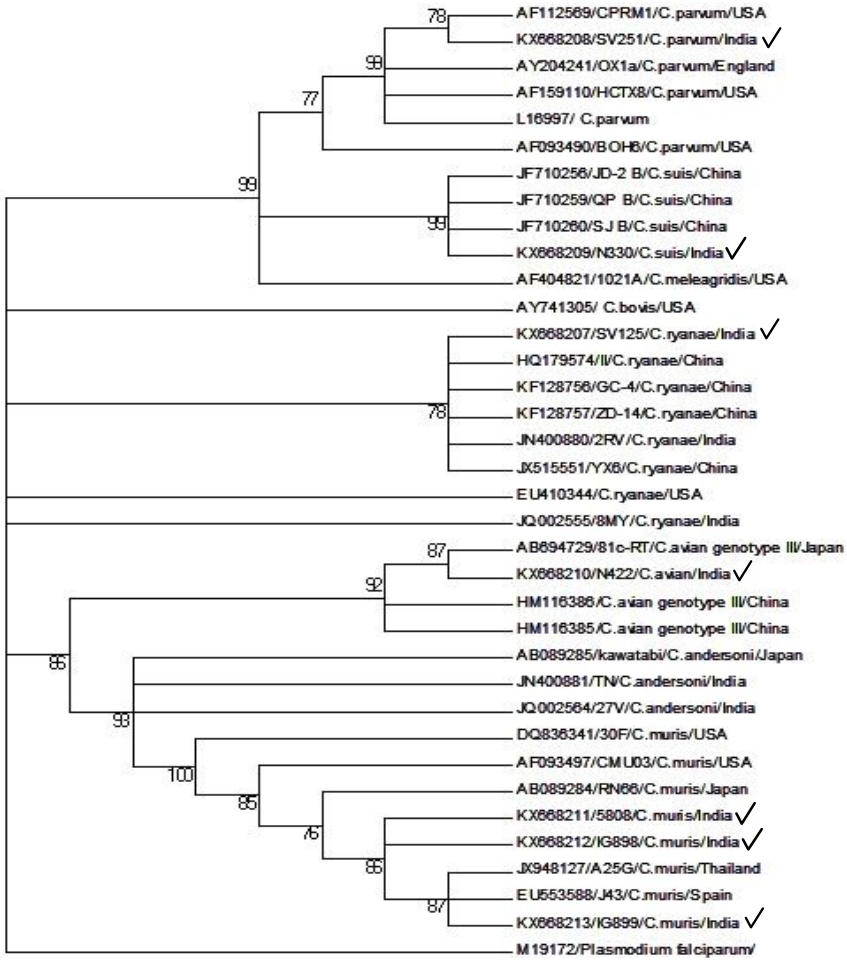


Fig 4: Phylogenetic relationship among *Cryptosporidium*isolates as inferred by neighbor-joining analysis of 18S rRNA gene.

Note: Accession numbers for reference sequences retrieved from GenBank. Numbers on branches are percent bootstrap values from 1000 replicates. The sequence for *P. falciparum*was used as an out group. Tick marked ones are generated sequences of the study.

CONCLUSION

The present study recorded the existence of *Cryptosporidium* infection in captive wildlife of India. To the best of author's knowledge, the current study is the first report focused on *Cryptosporidium* infection in captive wildlife from India. This pilot study data, alert the zoo veterinarians to take up the prophylactic measures against *Cryptosporidium* infection in India. Future studies are warranted to screen a wide range of wildlife across the country to analyze the *Cryptosporidium* genotypes found in relationship to their zoonotic potential.

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