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FIRST MOLECULAR REPORT OF ANAPLASMOSIS AND ITS ASSOCIATED RISK FACTOR IN CAPTIVE BIG CATS OF PAKISTAN

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ABSTRACT:
Anaplasmosis, a tick-borne disease affecting both animals and humans, has been investigated in domesticated big cats in Pakistan. This study aimed to determine the molecular prevalence of anaplasmosis and identify associated risk factors. Blood samples from 96 domesticated big cats were tested for the presence of Anaplasma using PCR, revealing a 15.63% infection rate. Nucleotide analysis indicated that the Anaplasma isolates were closely related to strains from Japan, Malaysia, the Philippines, and India. Logistic regression analysis identified significant risk factors, including gender, age, geographic area, vaccination history, previous exposure to flies, systemic diseases, recent transportation, exposure to domestic animals, body condition score, history of anemia, tick infestation, deworming history, house hygiene, tick control measures, housing type, jaundice, and appetite status. Infected cats exhibited significantly lower levels of erythrocytes, platelets, hemoglobin, and packed cell volume compared to healthy counterparts, indicating that anaplasmosis can lead to anemia and other hematological disorders. This study represents the first report of anaplasmosis in domesticated big cats in Pakistan, providing essential insights for the development of effective control strategies against this disease in these animals.

Keywords: Anaplasmosis, big cats, risk factors, Phylogenetic analysis, Hematological variables

1. INTRODUCTION

Many wild carnivore species are susceptible to severe and debilitating pathogens, with coinfections often exacerbating their clinical condition (Munson et al., 2008). The emergence and reemergence of these arthropod-borne pathogens have substantial implications for both human and veterinary medicine (Dantas-Torres et al., 2015).

Anaplasmosis is a critical tick-borne disease with a global distribution, particularly prevalent in

tropical and subtropical regions. This vector-borne illness is caused by various species of gram-negative obligate intracellular bacteria from the genus Anaplasma, affecting a broad range of hosts, including dogs, cats, horses, buffalo, cattle, goats, sheep, donkeys, cervids, and humans. The key Anaplasma species responsible for this disease include Anaplasma bovis, Anaplasma centrale, Anaplasma ovis, Anaplasma marginale, Anaplasma phagocytophilum, and Anaplasma platys. Anaplasmosis has significant implications for both veterinary and public health, as different Anaplasma species can infect humans and various animals, including pets like dogs and cats (Woldehiwet, 2010). In cats, Anaplasma species cause feline granulocytic anaplasmosis, a zoonotic illness that can also be transmitted to humans.

Though numerous vector-borne pathogens cause morbidity and mortality among domestic cats (Eberhardt et al., 2006), the exact role of some of these agents as disease causatives remains unclear. This gap in knowledge, alongside an incomplete understanding of the distribution and ecology of feline vector-borne diseases, has impeded the development of effective control measures to prevent infections in cats, other animals, and humans (Otranto et al., 2010). This study focuses on the molecular identification and associated risk factors of Anaplasma in captive big cats at various zoos and wildlife parks in Pakistan.

2. MATERIALS and METHODS

2.1. Study Design

This study was designed as a cross-sectional investigation to assess the prevalence, molecular characterization and risk factors analysis for Anaplasma in captive big cats (tigers and lions) in Pakistan. The study was conducted across various private and public zoos and wildlife reserves throughout the country.

2.2. Sample Collection

The study included 96 big cats, comprising both lions and tigers, from multiple public and private facilities. Blood samples were collected aseptically using the following procedures:

Blood Smears: Blood was drawn from ear tip punctures to create thick and thin blood smears, prepared in triplicates immediately after collection.

Blood Draw for Molecular Analysis: Additionally, 3 ml of blood was drawn aseptically from the tail vein using EDTA-coated vacutainers.

The collected samples were promptly transported to the Pet Center Laboratory, Department of Small Animal Clinical Sciences, University of Veterinary and Animal Sciences, Lahore. During transportation, the cold chain was maintained to ensure sample integrity. The blood samples in EDTA vacutainers were stored at -20°C until DNA extraction, which was initiated within 24-48 hours post-sampling.

2.3. Data Collection

A detailed data capture form was designed to record information on risk factor that included information about host, management, and environmental factors for each sampled big cat. This data was crucial for risk factor analysis.

2.4. Sample Processing

a) Microscopic Examination

Initial screening for Anaplasma spp. was conducted via microscopic examination of Giemsa-stained thin and thick blood smears under a 100X oil immersion lens. Samples were considered positive if intra-erythrocytic inclusion bodies resembling Anaplasma were observed. Positive samples from this screening were further subjected to molecular diagnostics and hemato-biochemical testing. A control group of healthy animals, negative for Anaplasma on both microscopy and PCR testing, was also included.

2.5. Molecular Identification of Anaplasma

a) DNA Extraction

DNA was extracted from the blood samples using the WizprepTM gDNA Mini Kit, following the manufacturer's instructions. The purity and concentration of the extracted DNA were measured using the NanoDrop method at a 260/280 nm wavelength, with an average yield of 40 ng/µl. The DNA samples were stored at -20°C until PCR analysis.

2.6. PCR Amplification of 16S rRNA Gene Fragment

The 16S rRNA gene fragment of Anaplasma spp. was targeted for PCR amplification using specific primers:

Forward primer: 5'-GGTACCYACAGAAGAAGTCC-3'
Reverse primer: 5'-TAGCACTCATCGTTTACAGC–3'
The PCR conditions followed the protocol described by Parola et al. (2003).

2.7. Sequencing of Positive PCR Products

PCR products displaying the specified bands were excised under UV illumination using a sterile blade and purified using the GeneAll® Expin™ Gel SV (102-150) extraction kit. The purified products were sent to 1st Base biological technology in Singapore for sequencing.

2.8. Sequence Analysis

Sequences were aligned with published Anaplasma sequences retrieved from the National Center for Biotechnology Information (NCBI) using BLAST. Multiple sequence alignment was conducted using the Clustal W method in BioEdit software. A phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA11) software, employing 1000 bootstrap replications and the maximum likelihood method to assess the percentage identity of the study sequences with retrieved sequences.

2.9. Statistical Analysis

Data were statistically analyzed using SPSS version 20.0. Risk factors were evaluated using the Chi-square test and multivariable logistic regression. Variables with a p-value less than 0.05 and an odds ratio greater than 1.00 were considered significant and indicative of potential risk factors associated with disease dynamics.

3. RESULTS

3.1. Prevalence and risk factors

This study investigated the prevalence of anaplasmosis in Felidae in Pakistan, revealing an overall prevalence rate of 15.63% (15/96). The highest prevalence was observed in white tigers (50%), followed by white lions (22.22%), royal Bengal tigers (20%), and African lions (13.04%). The differences in prevalence among these groups were not statistically significant ($p = 0.629$).

Tick infestation was identified as a significant risk factor for anaplasmosis. Animals with tick infestations showed a prevalence of 42.9%, compared to 15.7% in animals without tick infestations. Although this trend was notable, the results were not statistically significant ($p = 0.070$). Female animals exhibited a slightly higher prevalence (18.8%) compared to males (16.7%), but this difference was also not significant ($p = 0.789$). Age-related differences in infection rates were observed, with older animals showing higher prevalence: 66.7% in those over 25 years, 16.7% in the 16-25 year range, and 15.8% in those younger than 8 years. These differences approached significance ($p = 0.078$). Additionally, imported animals had a slightly higher prevalence (19%) compared to local ones (17.3%), though this was not significant ($p = 0.857$).

Animals with a vaccination history against other diseases had a higher prevalence (25.9%) compared to those without such a history (14.5%), but this difference was not statistically significant ($p = 0.187$). Interestingly, animals with a history of fly exposure had a significantly lower prevalence of anaplasmosis (9.6%) compared to those without (27.3%), with this difference being statistically

significant (p = 0.024).

Other factors such as a history of systemic disease (p = 0.434), recent transportation (p = 0.338), and exposure to other domestic animals (p = 0.056) showed no significant differences in prevalence. However, body condition score (BCS) was a significant factor, with higher prevalence in animals with BCS-1 (p = 0.003). The history of anemia was also highly significant, with a prevalence of 47.8% in anemic animals compared to 8.22% in non-anemic animals.

Housing conditions influenced prevalence as well; animals in hybrid housing showed higher prevalence compared to those in indoor or open housing, and this difference was significant (p = 0.01). Poor housing conditions were associated with higher prevalence, though not significantly (p = 0.187). Lastly, animals without a deworming history had a significantly higher prevalence of anaplasmosis (p = 0.002), and a history of jaundice was also significant (Table 1).

In the final logistic regression model, anemia and tick history emerged as the most significant risk factors for anaplasmosis in Felidae. Anemic animals had a 3.7% higher risk of anaplasmosis, while those with a tick history had a 2.9% higher risk. These findings underscore the importance of anemia and tick exposure as key factors in the prevalence of anaplasmosis in wild felids. Further research is needed to confirm these results and elucidate the relationship between anemia, tick exposure, and the development of anaplasmosis.

Table 1: Results of Chi-square test on various risk factors associated with anaplasmosis in domesticated big cats of Pakistan

Study Variable	Category	No. examined	No. positive	Prevalence25 (%)	OR	CI (95%)	P-value
Breed	African Lion	69	9	13.04	Referent	_	_
	Royal Bengal	10	2	20	1.7	0.3-9.1	P = 0.5560
	White Lion	9	2	22.2	1.9	0.3 to 10.6	P = 0.4629
	White Tiger	8	2	25	2.2	0.4 to 12.7	P = 0.3703
Gender	Female	48	9	18.8	1.2	0.403 to 3.295	P = 0.7893
	Male	48	8	16.7			
Age	Less than eighty	57	9	15.8	Referent		
	8 to 15 y	36	6	16.7	0.9375	0.3 to 2.9	P = 0.9108
	16 to 25 y	3	2	66.7	0.0938	0.007 to 1.146	P = 0.0639
Area	Imported	21	4	19	1.1222	0.3-3.8	P = 0.8557
	Local	75	13	17.3			
Vaccine history	Yes	27	7	25.9	2.065	0.69 to 6.14	P = 0.1927
	No	69	10	14.5			
Previous History of Flies	Yes	52	5	9.6	0.2837	0.0911 to 0.88	P = 0.0297
	No	44	12	27.3			
Other Systemic Diseases	Yes	7	2	28.6	1.97	0.34 to 11.14	P = 0.4416
	No	89	15	16.9			
Transportation History in the Last Year	Yes	25	6	24	1.7225	0.5617 to 5.2823	P = 0.3416
	No	71	11	15.5			
Domestic Animal Exposure	Yes	14	5	35.7	3.2407	0.9256 to 11.34	P = 0.0659
	No	82	12	14.6			
Body Condition Score	1	14	7	50	Referent		
	2	77	9	11.7	7.5556	2.14 to 26.56	P = 0.0016

	3	5	1	20	4.0	0.35 to 45.38	P = 0.2633
Anemia	Yes	23	11	47.8	10.23	3.1796 to 32.95	P = 0.0001
	No	73	6	8.22			
History of Tick Infestation	Yes	15	5	33.3	2.875	0.8351 to 9.8976	P = 0.0941
	No	81	12	14.8			
Tick Control	Yes	92	15	16.3	0.1948	0.0254 to 1.49	P = 0.1154
	No	4	2	50			
Tick Infestation	Yes	7	3	42.9	4.0179	0.8095 to 19.9414	P = 0.0889
	No	89	14	15.7			
Housing Type	Open	43	2	4.7	Referent		
	Indoor	15	4	26.7	0.1341	0.0217 to 0.8306	P = 0.0308
	Hybrid	38	11	28.9	0.1197	0.0246 to 0.5831	P = 0.0086
House hygiene	Poor	8	4	50	Referent		
	Fair	87	13	14.9	5.692	1.2624 to 25.6663	P = 0.0236
	Good	1	0	0	3.0	0.0946 to 95.1760	P = 0.5334
Deworming history	Yes	87	12	13.8	0.1280	0.0301 to 0.5452	P = 0.0054
	No	9	5	55.6			
Jaundice	Yes	15	8	53.33	9.1429	2.6764 to 31.2328	P = 0.0004
	No	81	9	11.1			
Appetite status	0	1	0	0	Referent		
	1	18	8	44.4	0.4118	0.0148 to 11.4578	P = 0.6011
	2	16	7	43.8	0.3778	0.0133 to 10.7451	P = 0.5687
	3	61	2	3.3	23.6000	4.3622 to 127.6793	P = 0.0002

3.2. Molecular Characterization

Detection and Characterization of Anaplasma spp. in captive Big Cats of Pakistan

This study represents the first confirmation of Anaplasma spp. presence in domesticated big cats in Pakistan using molecular methods. The 16S rRNA gene fragment of Anaplasma spp. was successfully sequenced from PCR products derived from locally collected samples. Gradient PCR was employed to optimize the amplification of the 16S rRNA gene fragment specific to Anaplasma spp. (Figure 1). The use of gradient PCR allowed for the identification of the optimal annealing temperature, ensuring specific and efficient amplification of the target gene fragment.

3.3. Phylogenetic Analysis

The sequencing results revealed distinct clusters among the Anaplasma isolates. Notably, the isolates Anaplasma 11, 8, and 6 formed a separate cluster, while Anaplasma 18 and 22 constituted another distinct group. These clusters were found to exhibit a degree of similarity to an isolate of A. capra. The phylogenetic analysis indicates that while these Anaplasma spp. isolates share similarities with A. capra, they also demonstrate distinct genetic characteristics that warrant further investigation (Figure 2).

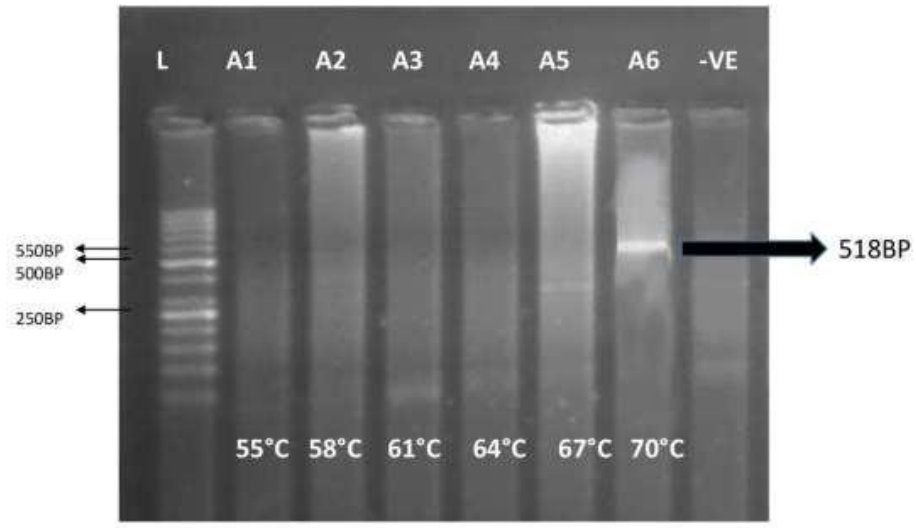

Figure 1: Gradient PCR For Anaplasma

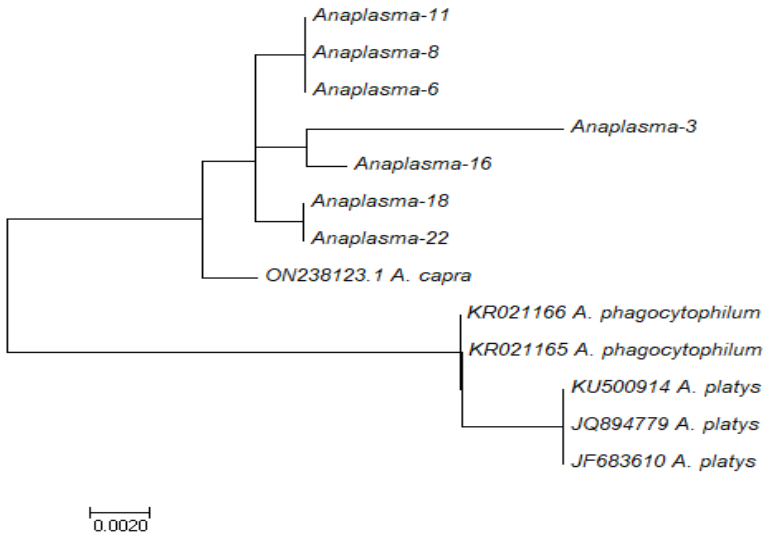

Figure 2: Phylogenetic analysis indicates that Anaplasma spp. isolates share similarities with A. capra

4. DISCUSSION

Tick-borne pathogens are increasingly significant in both veterinary and human medicine (Dantas-Torres et al., 2012). Particularly, pathogens affecting domestic cats have garnered significant research interest. Feline anaplasmosis is an emerging disease, with its prevalence varying widely across different regions due to factors such as geographical climate, vector distribution, infection status, socioeconomic conditions, and the type of PCR utilized (Conventional PCR, Real-time PCR, Nested PCR). Although anaplasmosis in domestic cats has not been previously reported in Pakistan, other feline tick-borne diseases like babesiosis have. This study marks the first molecular diagnosis of Anaplasma spp. in large feline species in Pakistan, analyzing assumed risk factors associated with disease dynamics and conducting phylogenetic analysis of local isolates.

In this study, the overall prevalence of anaplasmosis in domesticated big cats was found to be 15.63% based on PCR results. This prevalence is notably higher than that reported by André et al. (2012), who found an 8% molecular prevalence of Anaplasma spp. in free-roaming cats in São Paulo, Brazil. Other studies also reported lower prevalence rates: Maia et al. (2014) found a 5.4% prevalence in Southern Portugal, and molecular prevalence rates of A. phagocytophilum were 1%, 0.4%, and 0.9% in Spain, Southern Germany, and Korea, respectively (Bergmann et al., 2015; Lee et al., 2018; Tabar et al., 2008). Additionally, Attipa et al. (2017) reported a 0.6% prevalence of A. platys in Cyprus cats,

and a study in Luanda, Angola, found a 1% prevalence of A. bovis in domestic cats using PCR and DNA sequencing.

The higher prevalence of anaplasmosis observed in this study compared to previous research can be attributed to several factors. Pakistan's tropical and subtropical climate, which is hot and humid, provides an ideal environment for ticks (Ali et al., 2019; Batool et al., 2019), thereby increasing the risk of tick-borne diseases. Unlike previous studies, this research applied specific inclusion criteria, selecting cats based on various risk factors such as gender, age, area, vaccination history, previous history of fly exposure, other systemic diseases, transportation history, domestic animal exposure, body condition score, anemia, tick infestation history, tick control measures, housing type, house hygiene, deworming history, jaundice, and appetite status. Only cats presenting with tick infestation and clinical signs of fever, lethargy, anemia, and dehydration were included, which likely contributed to the higher observed prevalence.

The lion (Panthera leo) faces extinction risks due to human activities such as poaching, habitat destruction, and disease spread. Tick-borne infections are known to affect lions, potentially contributing to population declines. Torina et al. (2007) reported the presence of C. burnetii, spotted fever group Rickettsia sp., and A. phagocytophilum in 50%, 20%, and 10% of lions, respectively, without clinical indications of infection.

This study represents the first molecular confirmation of Anaplasma spp. in domesticated big cats in Pakistan, marking a significant advancement in our understanding of these pathogens in non-traditional hosts. The implementation of gradient PCR was critical in optimizing the amplification of the 16S rRNA gene fragment specific to Anaplasma spp. This optimization is crucial for the reliability of PCR-based detection methods, as it reduces the likelihood of non-specific amplification and enhances the sensitivity of the assay (Zhou et al., 2010). The phylogenetic analysis of the sequencing results revealed distinct clusters among the Anaplasma isolates, indicating a degree of genetic diversity within the sampled population. Notably, the isolates Anaplasma 11, 8 and 6 formed a separate cluster, while Anaplasma 18 and 22 constituted another distinct group. This clustering pattern suggests that multiple Anaplasma spp. may be circulating within the captive big cat population in Pakistan. Interestingly, these clusters were found to exhibit a degree of similarity to an isolate of A. capra, a recently identified species within the genus Anaplasma (Yang et al., 2016). While the Anaplasma spp. isolates from our study share similarities with A. capra, they also demonstrate distinct genetic characteristics. This finding warrants further investigation to elucidate the potential zoonotic implications and epidemiological significance of these isolates.

The identification of Anaplasma spp. in big cats also raises important questions about the potential vectors and transmission dynamics of these pathogens in captive environments. Previous studies have documented the presence of Anaplasma spp. in various tick species, which are known vectors of these pathogens (Rar and Golovljova, 2011). It is plausible that ticks could be playing a role in the transmission of Anaplasma spp. among captive big cats, highlighting the need for integrated vector management strategies in zoological settings.

CONCLUSION

Findings of this study provide significant insights into the genetic diversity of Anaplasma spp. in domesticated big cats in Pakistan. The successful amplification and sequencing of the 16S rRNA gene fragments highlight the utility of molecular methods in detecting and characterizing Anaplasma spp. in non-traditional hosts. The observed clustering of isolates suggests potential genetic variability and host-specific adaptations within the Anaplasma spp. present in these big cats.

Further research is needed to explore the epidemiological implications of these findings and to understand the potential impact of Anaplasma spp. infections on the health of domesticated big cats. Additionally, the relationship between these isolates and other known Anaplasma species, such as A. capra, should be investigated to elucidate the evolutionary dynamics and host range of this genus.

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