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Active surveillance and associated epidemiological risk factors of caprine paratuberculosis in selected district of Odisha, India

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Abstract

Caprine paratuberculosis (PTB) is a progressive, debilitating and production-limiting disease that causes significant economic losses and raises public health concerns. The goal was to study active surveillance and associated epidemiological risk factors of caprine PTB in selected district of Odisha, India. The 818 goats of various ages, sexes and breeds were randomly screened in ten different districts for a year based on history, clinical signs and fecal smear examination using the Ziehl-Neelsen stain, yielding an overall prevalence of 38.75%, with clinical and sub-clinical PTB at 8.06 and 30.68%, respectively. A molecular tool, IS900 polymerase chain reaction, was also used to confirm the disease. With *Mycobacterium avium* subsp. *paratuberculosis* (MAP) bacilli and endoparasite infections, the majority of affected goats (69.08%) were low shedders. Puri coastal district had the highest prevalence (52.29%) followed by Sambalpur (48.61%), while Khordha had the lowest prevalence (26.41%). Caprine PTB was more common in goats over 2 years old (51.23%), in the Ganjam breed (42.30%), in females (39.17%) and in goats housed on earthen floors (55.83%) according to chi-square analysis. The current study concluded that higher (30.68%) observations of subclinical PTB were cause of real concern due to its insidious spread as well as its zoonotic significance with potential human consequences, which requires immediate attention at all levels. Because of the public health importance of this hidden killer disease, the current findings would be useful in developing a roadmap for implementing prevention and control policies, prompting provision for adequate funding with elaborative research.

Introduction

Caprine paratuberculosis (PTB) is a progressive, debilitating and production-limiting disease that causes significant economic losses to marginal farmers including herd loss due to high mortality, increased premature culling risk, lower reproductive efficiency, compromised growth rate and decreased milk yield.[1] It is a chronic condition that can go unnoticed for years referred to as a "hidden threat"[2] or a "silent slayer",[3] caused by a gram positive, acid-fast bacillus known as *Mycobacterium avium* subsp. *paratuberculosis* (MAP).[4] The feces of infected animals contaminate the environment and their prolonged persistence in harsh conditions complicates disease pathogenesis and causes a public health problem.[5] Living with these animals poses a public health risk for poor people because MAP has been isolated for Crohn's disease patients.[6]

Assam reported the first case of PTB in India.[7] In Odisha, Biswal *et al*.[8] recorded 68.19% prevalence of MAP. Clinical signs observed after a prolonged incubation period include lethargy, emaciation, prolonged weight loss, weak-ness and diarrhea most commonly in adults of more than 2 years of age.[9] Various epidemiological factors have a significant impact on the pathology and pathogenesis of this disease. Aside from clinical signs, the normal shedding of MAP bacilli through feces aids in routine field screening of goats.

Due to the time required for confirmation via fecal culture as well as the lack of a specific diagnostic assay and treatment in sub-clinically affected animals, PTB

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prevention and control in small ruminants is a difficult task.[3] Even though PTB is considered endemic in India,[10] no comprehensive study has been conducted to determine the prevalence of MAP in small ruminants in Odisha, where goat farming is important in the rural economy for livelihood sustenance. The government of Odisha, an eastern state of the Republic of India, encouraged goat farming as a primary source of livelihood in rural areas through the state women self-help groups which had the bitter experience of frequent natural calamities resulting in complete destruction of the economic backbone through massive crop destruction. Despite several setbacks in terms of job creation and food security, the goat husbandry sector is the only one that can provide a consistent income. Goats are also used in religious rituals, marriage ceremonies in rural areas of the state and as raw cash in the exchange of goods in times of need. Odisha goat (1.84%) and sheep (19.10%) populations have steadily declined since the 19th and 20th livestock censuses, requiring a thorough review. There is lack of knowledge among farm owners on this economically significant disease, because early detection is critical to halt its spread in flocks. Furthermore, the lack of knowledge about MAP, as well as the limited studies conducted in Odisha, may be a factor in its rampant spread through communal grazing and animal markets. The study research recommendations will be useful in updating all stakeholders in the small ruminant industry so that appropriate policy decisions can be made in the future to combat this deadly slow spreading disease among flocks. The main aim of the present study was to know the occurrence of caprine PTB by the active surveillance and its associated epidemiological risk factors in selected district of Odisha, India.

Materials and Methods

Animal, samples and study area description. From December 2020 to November 2021, a thorough investigation was conducted in screening goats against PTB in ten different districts of Odisha (Fig. 1), an eastern state of India considered endemic for PTB, located between 81° 27' E - 87° 29' east longitude and 17° 49' N - 22° 34' north latitude with a population of nearly 1.30 million sheep and 6.39 million goats, respectively. Cross sectional study was conducted to estimate the prevalence and associated risk factors for caprine PTB. The goat owners who showed willingness to participate to screen their animals were included in this study. In ten different districts, 818 goats of various ages, sexes and breeds were randomly screened. Samples such as feces were collected in sterile containers from randomly selected goat herds and transported to the testing laboratory in a Thermocol Box with frozen Gel Fack and stored in a deep freezer (– 80.00 ˚C) until further use.

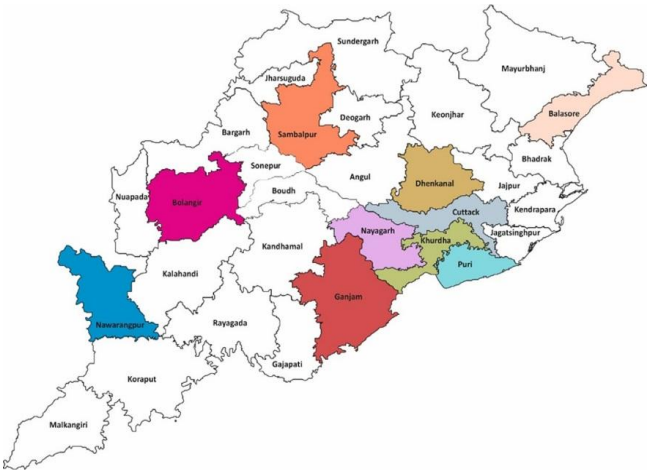

Fig. 1. Showing sites of sample collection from different districts of Odisha, India.

Epidemiological risk factors. Age (< 1 year, 1 - 2 year, > 2 year), sex (female and male), breed (Ganjam, Blak Bengal, Raighar), floor type (cement, earthen, bamboo-netting), season (rainy, summer, winter) and district (Khordha, Dhenkanal, Balasore, Ganjam, Puri, Sambalpur, Nawarangapur, Navagarh, Cuttack, Bolangir) were considered as the epidemiological risk factors and data were collected from farm owners at their doorstep.

Diagnosis of caprine PTB. Diagnosis of caprine tuberculosis were carried out based on clinical sings (loss of appetite, weak and emaciated, rough hair coat, weight loss, softening of feces or diarrhea, anemia, intermandibular oedema in few goats), fecal sample examination by Ziehl-Neelsen (Z-N; HiMedia, Mumbai, India) staining method and confirmatory diagnosis by IS900 polymerase chain reaction (PCR).

Detection of MAP in fecal sample by Z-N staining method. Ziehl-Neelsen stain was used to detect acid-fast bacteria in fecal samples collected from the rectum of all 818 goats.[11] In brief, 2.00 g of fecal samples were homogenized in 3.00 - 4.00 mL of sterile normal saline solution, then diluted with 7.00 - 8.00 mL of normal saline solution and centrifuged at 3,500 rpm for 45 min to perform Z-N staining. The supernatant was discarded and a thin fecal smear was prepared, air dried, gently heated and stained with Z-N staining according to the manufacturer's instructions before being observed microscopically using oil immersion under 100× magnification. Based on the number of MAP in the microscopic area, the goats were classified as low (1 - 3 bacilli in one field *i.e.*, +1), moderate (4 - 5 bacilli in one or more field *i.e.*, +2) or high shedders (10 or > 10 bacilli in one field *i.e.*, +3).

Confirmation of MAP by genome detection. The DNA was extracted from fecal samples (200 mg) in accordance with the manufacturer's instructions (Qaim fast DNA mini kit; Qiagen, Hilden, Germany). The quality

and quantity of extracted DNA were measured using a NanoDrop™ 1,000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and stored at – 80.00 ˚C until further use. The MAP genome detection was performed using PCR on DNA extracted from fecal samples positive for MAP by Z-N staining.[12] In brief, PCR was performed using MAP specific primers forward (5'-GAAGGGTGTTCG GGGCCGTCGCTTAGG-3') and reverse (5'-GGCGTTGAGG TCGATCGCCCACGTGAC-3') and PCR amplified products (400 bp) were electrophoretically separated and stained with 2.00% Ethidium Bromide on a 1.00% agarose gel (Bio-Rad, Hercules, USA). A 5.00 µL of extracted DNA from a tissue sample was amplified in a reaction mixture that included 12.50 µL of 2.00 X PCR master buffer, 1.00 µL of 25.00 X enzyme mix, 1.00 µL of 10.00 pmol each of forward and reverse primers, and the final volume of the reaction mixture was adjusted up to 25.00 µL with nuclease-free water. The parameters for PCR reaction condition start with an initial denaturation step at 95.00 ˚C for 5 min, 35 cycles of denaturation at 95.00 ˚C for 30 sec, an annealing step at 54.00 ˚C for 45 sec, an extension step at 72.00 ˚C for about 45 sec and a final extension step at 72.00 ˚C for 5 min.

Statistical analysis. Statistical analysis of data was performed in SAS Software (version 7.0; SAS Institute, Cary, USA) using the Chi-square ($\chi 2$) and Student's *t*-test at $p \leq 0.05$.

Results

Clinical signs. Only 66 (20.82%) out of 317 goats tested positive for caprine PTB showed clinical signs including reduced body weight, progressive emaciation despite a good appetite, recurrent pasty feces and/or greenish diarrhea mostly full of bubbles, rough hair coat, poor body condition, marked depression, increased hair loss, soiling of perineum and sub-mandibular swelling (Fig. 2). All other goats (n = 251, 79.18%) did not show any typical clinical signs during the investigation, however, their fecal sample tested positive for acid fast bacilli through Z-N staining along with poor body condition and a recent history of irregular diarrhea indicated clinical form of PTB.

Detection of MAP in fecal sample by Z-N staining method and confirmatory diagnosis of MAP by genome detection. Total number of 317 fecal samples were found positive for MAP by Z-N staining method followed by confirmatory diagnosis by MAP genome detection. Based on number of MAP in oil emersion microscopic area, the goats were classified as low, moderate or high shedders. There was a high occurrence of low shedders (n = 219, 69.08%) followed by moderate shedders (n = 64, 20.18%) and the least number of high shedders (n = 34, 10.72%; Fig. 3). All MAP positive samples by Z-N staining were also found positive by PCR method. Positive PCR results for MAP were found as 400 bp products using specific oligo IS900 PCR primers (Fig. 4).

Fig. 2. Showing goat status during the investigating of caprine paratuberculosis (PTB). **A)** illustrates a goat flock with moderate body condition, **B)** goat with reduced body weight and progressive emaciation, **C)** greenish pasty feces/ diarrhea in PTB affected goat, **D)** cement flooring in goat shed affected with PTB, **E)** marked depression in PTB affected goats, and **F)** external soiling of perianal regions and matted hairs in PTB affected goats.

Prevalence of caprine PTB. The current study reported that 317 goats were positive for caprine PTB with an overall prevalence of 38.75%. Prevalence of clinical and sub-clinical PTB were 8.06 and 30.68%, respectively. District wise prevalence recorded the highest occurrence in Puri district (n = 57, 52.29%) followed with Sambalpur (n = 35, 48.61%), Balasore (n = 36, 45.00%), Dhenkanal (n = 34, 39.53%), Cuttack (n = 32, 37.20%), Ganjam (n = 45, 34.88%), Nawarangapur (n = 24, 34.78%), Nayagarh (n = 19, 32.20%), Bolangir (n = 21, 28.00%) and the least prevalence in Khordha (n = 14, 26.41%) with significant difference ($p \leq 0.05$).

Epidemiological risk factors. Information on epidemiological risk factors for caprine PTB prevalence is presented in Table 1. Chi-square analysis revealed that

disease occurrence was significantly higher in adult goats older than 2 years (n = 208, 51.23%) than in those younger than 2 years (n = 109, 40.82%; $p \leq 0.05$). The does (n = 291, 39.16%) had a higher incidence than the bucks (n = 26, 34.67%). The current study recorded a significant relationship between disease occurrence and breed ($p \leq 0.05$), with the Ganjam breed having the highest incidences (n = 187, 42.30%), the Black Bengal breed having the second highest (n = 106, 35.93%), and the Raighar breed having the lowest (n = 24, 29.63%). The PTB was the highest in goats reared on earthen flooring (n = 177, 39.95%) followed by bamboo matting (n = 57, 39.04%) and the lowest in cement flooring (n = 83, 36.24%) but was not significantly different ($p \geq 0.05$). Similarly, occurrence of caprine PTB did not differ significantly in male and female goats and it did not depend on seasons ($p \geq 0.05$).

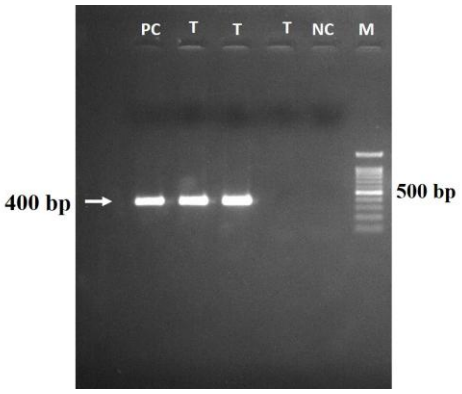

Fig. 4. Confirmation of *Mycobacterium avium* subsp. *paratuberculosis* MAP by detection of MAP specific genome. Agarose gel electrophoresis of PCR products showing presence of distinct bands of positive control (PC), test samples (T), negative control (NC), and 100 bp molecular weight marker (M).

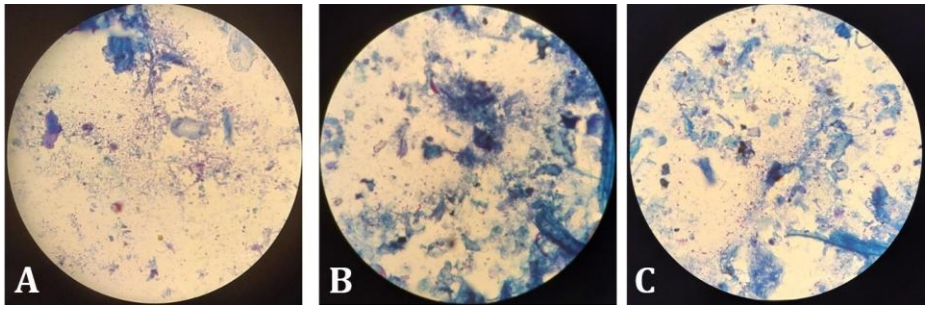

Fig. 3. Detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in fecal smear examination through Ziehl-Neelsen staining and classified into **A)** low shedder, **B)** moderate shedder and **C)** high shedders of MAP in feces of goat.

Table 1. Epidemiological risk factors in occurrence of caprine paratuberculosis.

Factor	Category	No.	Positive (%)	$\chi 2$	DF	*p*-value
Age	< 1 year	145	0[a]	118.860	2	0.00001*
	1 - 2 year	267	109(40.82%)[b]			
	> 2 year	406	208(51.23%)[c]			
Sex	Female	743	291(39.16%)	0.580	1	0.446**
	Male	75	26(34.67%)			
Breed	Ganjam	444	187(42.30%)[b]	6.180	2	0.045*
	Black Bengal	295	106(35.93%)[ab]			
	Raighar	81	24(29.63%)[a]			
Floor type	Cement	229	83(36.24%)[a]	0.882	2	0.643**
	Earthen	443	177(39.95%)[a]			
	Bamboo-netting	146	57(39.04%)[a]			
Season	Rainy	330	128(38.80%)[a]	0.001	2	0.999**
	Summer	276	107(38.80%)[a]			
	Winter	212	82(38.70%)[a]			
District	Khordha	53	14(26.41%)[a]	22.180	9	0.008*
	Dhenkanal	86	34(39.53%)[ab]			
	Balasore	80	36(45.00%)[ab]			
	Ganjam	129	45(34.88%)[ab]			
	Puri	109	57(52.29%)[b]			
	Sambalpur	72	35(48.61%)[ab]			
	Nawarangapur	69	24(34.78%)[ab]			
	Nayagarh	59	19(32.20%)[ab]			
	Cuttack	86	32(37.20%)[ab]			
	Bolangir	75	21(28.00%)[a]			

*$p \leq 0.05$ means significant **$p > 0.05$ means non-significant.
[ab] Values with different superscripts differ significantly ($p \leq 0.05$)

Discussion

This was the first in-depth investigation conducted in Odisha, India, with the goal to know the prevalence of caprine PTB and its associated epidemiological risk factors. Our study clinical findings agreed with those of Bonelli *et al.*[13] and Scherrer *et al*.[9] More endoparasite infections may be linked to poor management which is common in rural unorganised farms as well as improper metabolic maintenance at various stages of growth which puts more stress on the body and makes it more susceptible to various types of infections.[14] In current study, high occurrences of low shedders of MAP bacilli followed by moderate shedders and the least numbers belonging to high shedders were recorded and these criteria of shedders were important for screening of goats into clinical as well as sub-clinical caprine PTB. More MAP bacilli shedding in feces was indicative of different stages of infection.[15,16] In the absence of specific clinical signs, fecal smear examination using acid-fast staining is quite reliable for screening goats even at an early stage of infection in order to limit the spread by isolating or culling positive animals.[13,17] Individual animal genetic make-up as well as individual goat immune responses to various stages of infection may influence the type of bacilli through fecal shedding.[18] Typical clinical signs of progressive emaciation, recurrent diarrhea, alopecia, and submandibular oedema are seen in goats with clear and intense proliferations in the intestine resulting in chronic granulomatous enteropathy and increased shedding through stools or pasty feces in severely affected goats.[10] Early stages of MAP infection are associated with frequent interactions with cell-mediated immune responses, so they are cleared from the body with very little shedding through feces.[19]

The current study recorded an overall prevalence of caprine PTB of 38.75%, with clinical and sub-clinical PTB at 8.06% and 30.68%, respectively. This finding was consistent with earlier workers.[10,20] The introduction of a silent spreader into goat herds as well as a lack of public awareness about the disease may all contribute to PTB prevalence as does the etiological agents long persistence in the environment.[21] Subclinical infections are becoming more common due to a longer incubation period, complex pathogenesis of intracellular bacilli and lack of a reliable diagnostic field test with high sensitivity and specificity for early detection of subclinical infection.[22] Clinically affected goats with PTB gradually develop emaciation and reduced lean body weight because of malabsorption, therefore, the owner prefers to sell it in the market to make a profit rather than incurring unnecessary veterinary expenses.[23]

Due to the geographical distribution of MAP and their ease of persistency in various agroclimatic conditions, chi-square analysis revealed a significant relationship between the occurrence of caprine PTB and different districts.[24] All three MAP-increased districts were coastal which had some internal implications for environmental persistence[25] and warranted further investigation. Because MAP spreads from one animal to another via the teat of an infected nanny or ingestion of manure contaminated pasture, water, supplements, or hay,[18] the low prevalence in Khurda (n = 14, 26.41%) district could be due to a lack of extensive grazing systems for goat herds in this highly urbanised district as well as a smaller sample size. Increased incidences in older age groups were consistent with the disease long incubation period.[16] Infected animals with PTB might not be able to show active clinical symptoms even at 2 years of age, thus, in our study, we found a higher occurrence of MAP in goats older than 2 years.[26]

Increased incidences in the Ganjam breed of goats were linked to a larger sample size as well as farmers domesticating them for a longer period for the purpose of meat, milk and good quality male bucks.[27] Due to their early slaughter for good quality meat in Odisha, Black Bengal goats had a lower incidence, whereas, Raighar goats had a lower incidence due to genetic makeup and a smaller sample size. Higher MAP incidences in Ganjam goats might be attributed to their larger size and the practise of "Gola communities" keeping large sized herds rather than small sized Black Bengal goats.

The larger sample size could be attributed to higher female affections, as farm owners were more interested in keeping does for production purposes. Female animals were more stressed than male animals due to pregnancy, lactation and parturition, all of which contributed to increased disease incidences.[28]

Goats housed on earthen floors had higher rates of affection which could be attributed to MAP bacilli increased persistence due to unsanitary conditions. According to Singh *et al*.,[10] MAP bacilli fecal shedding remained in soils for long periods of time. Because of the frequent clearing in the extensive management system, cement and bamboo flooring had lower disease affections due to less etiological agent persistence in these floors.[29] Bacteria bioaccumulation from clinical and subclinical shedders may result from frequent urination and defecation in goat sheds with earthen flooring. Cement and bamboo net flooring which are commonly used in large-scale goat rearing management systems are associated with regular floor washing and clearing by farmers resulting in a lower chance of MAP bacilli settling.[16] Bacteria can grow quickly in unsanitary and wet environments during the rainy season and they can easily spread among flocks due to increased confinement in sheds due to rain outside which complicates the flooring conditions resulting in more and more bacteria bioaccumulation.[30] Dry summer weather as well as reduced contamination of flooring due to dryness might be associated with a lower disease affection.[24]

The best diagnostic method for caprine PTB, polymerase chain reaction (PCR), can be obtained within three days of sample collection allowing for a rapid screening response in a suspected herd.[17] The MAP bacilli shedding in greater quantities through fecal samples of infected goats at various stages of infection considered an important clinical sample for DNA extraction and molecular confirmation through PCR always gives 100% sensitivity and specificity in all samples.[1]

In present study, there was a high incidence of PTB (38.75%) in Odisha indicating that regular screening of faeces using acid-fast staining could be used in the field to arrive at a fast-reliable preliminary diagnosis. Because of its insidious spread and zoonotic significance with potential human consequences, higher (30.68%) observations of subclinical PTB were cause of real concern. Detection of caprine PTB by non-invasive approach using fecal sample using Z-N staining and confirmation by molecular-based techniques like PCR might be used to assess disease severity and plan the treatment regimen. The current study concluded that the disease in Odisha was precipitated by poor management and various epidemiological risk factors, and that it requires immediate attention at all levels. Because of importance of the state public health, the current study findings would be useful in developing a roadmap for implementing prevention and control policy measures for this hidden killer disease, prompting provision for adequate funding with elaborative research.

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Conflict of interest

With respect to publication of this research findings, the authors declares that there are no conflict of interest.

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