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Risk Factors Associated with Prevalence of Zoonotic Tuberculosis in Small Ruminants in Chhattisgarh, India

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ABSTRACT

This study carried out in 2021-22 and 2022-23 at the College of Veterinary Science & A.H., Anjora, Durg, and ICAR-NIBSM, Baronda, Raipur, Chhattisgarh, India focused on examining molecular detection and determining risk factors associated with infections of Bovine tuberculosis (BTB) in small ruminants within the Chhattisgarh region. Zoonotic tuberculosis, a global threat emerging from the *Mycobacterium tuberculosis complex*, impacts humans, livestock, and wildlife. A total of 795 samples from small ruminants, including blood, aborted fetuses, vaginal swabs, and placental tissues, were collected. DNA extraction and multiplex PCR targeting *Mycobacteria* RD1 and RD4 genes were performed. Statistical analyses explored individual animal-level and herd-level risk factors using SPSS. Results revealed a 0.75% prevalence of BTB in Small ruminants. Herd-level analysis demonstrated 2.6% prevalence. The statistical analysis assessing individual animal-level risk factors associated with BTB prevalence showed no significant associations with species, age, sex, or sample type. Herd-level risk factors included the number of small ruminants (OR=1.158, p =0.003) and introducing new animals (OR=1.090, p=0.034). In a multivariable logistic regression analysis, the variable "Number of small ruminants kept" emerged as a significant predictor (OR=1.155, p=0.004).Other factors like vaccination, biosecurity measures, and history of reproductive disorders did not reach statistical significance. This study provides comprehensive insights into the multifaceted risk factors associated with zoonotic tuberculosis transmission in small ruminants. The findings underscore the importance of ongoing research to formulate effective control strategies considering the nuanced nature of BTB.

KEYWORDS: Chhattisgarh, molecular detection, *Mycobacteria*, risk factor, small ruminants

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1. INTRODUCTION

Zoonotic tuberculosis, stemming from the *Mycobacterium tuberculosis complex*, stands as a formidable threat that transcends species boundaries, affecting humans, livestock, and wildlife worldwide. At the heart of this complex lies *Mycobacterium bovis*, a constituent of notable significance with far-reaching implications, particularly in international trade in animals and related products.

Despite the implementation of a global eradication strategy for zoonotic tuberculosis, the disease's endemic nature persists in numerous countries, including India, where the absence of a specific tuberculosis eradication control program for animals exacerbates the challenges. Zanardi et al. (2013) emphasized the continued endemicity of tuberculosis, specifically in India, where the goat population exceeds 135 million, playing a vital role in providing protein and textile resources.

Zoonotic TB, also recognized as bovine TB, is transmitted to humans primarily through the consumption of raw dairy products, occupational exposure to livestock, and less commonly through the ingestion of untreated meat products (Ayele et al., 2004). Neglected for decades in many developing regions, zoonotic tuberculosis remains a significant concern, recognized as a priority by the World Health Assembly since the 1950s (Mableson et al., 2014). The various identified key risk factors, including previous contact with active TB cases, consumption of raw milk, and HIV infection, contributing to the perpetuation of this neglected yet impactful disease (Cleaveland et al., 2007; Bapat et al., 2017). India's status as a major contributor to the world's meat and milk production, highlighting the estimated 7.3% prevalence of bovine TB among farm and dairy cattle in the country (Ramanujam and Palaniyandi, 2023). In 2017, a collaborative effort involving the World Health Organization (WHO), the World Organization for Animal Health (OIE), the Food and Agriculture Organization (FAO), and the International Union against Tuberculosis and Lung Disease resulted in the launch of a zoonotic tuberculosis roadmap. This roadmap, aimed at addressing the intersection of tuberculosis in animals and humans, underscores the importance of enhancing scientific evidence through comprehensive data collection. Additionally, it targets the reduction of zoonotic TB transmission at the human-animal interface to ensure food safety, emphasizing collaborative engagements (Gompo et al.,2020; Dean et al., 2018).

Among various diagnostic methods, PCR has emerged as a promising and superior technique for diagnosing infectious diseases caused by fastidious or slow-growing bacteria. However, it is noteworthy that most PCR assays amplify fragments from the *Mycobacterium tuberculosis complex*, posing a challenge in distinguishing infections caused by *M. tuberculosis* from those caused by *M. bovis.* To address this issue and differentiate species within the *Mycobacterium tuberculosis complex* (MTBC), the RD1 and RD4 duplex PCR method is employed. This method, has demonstrated high reliability, enabling the detection of various species in a single reaction (Taylor et al., 2007; Halse et al., 2010).

The significance of different risk factors in *M. bovis* transmission depends on the prevalence and magnitude of other risks. Therefore, understanding the interplay of these risks is crucial for comprehending the epidemiology of the disease. This comprehension is essential for formulating effective policies and strategies for eradicating and controlling bovine tuberculosis in small ruminants.

The present study aims to ascertain the prevalence of Bovine Tuberculosis (BTB) among small ruminants in Chhattisgarh and to evaluate potential risk factors associated with infection and characterization of the species of *Mycobacteria* present in small ruminants.

2. MATERIALS AND METHODS

2.1. Sampling methodology

In Chhattisgarh, a comprehensive study aimed at molecularly detecting *Mycobacteria* species in small ruminants involved the collection of 750 blood samples, 33 aborted foetus samples, 9 vaginal swabs, and 3 placental tissues, from 756 goats and 39 sheep. The animal distribution included 48 males and 747 females. The age distribution ranged from 1 to 4 years, with specific counts for each age category. The samples were collected from diverse districts across Chhattisgarh, providing a comprehensive dataset for the investigation.

2.2. Molecular detection of Mycobacteria spp. from small ruminants

For molecular detection DNA extraction from collected samples utilized a QIAGEN DNeasy Blood and Tissue Kit. The process involved labeling microcentrifuge tubes, adding Proteinase K and blood/tissue samples, adjusting volumes with PBS, and incubating at 56°C. Ethanol and Buffer AL were sequentially added, and the mixture was processed using DNeasy Mini spin columns. Subsequent steps included washing with Buffer AW1 and Buffer AW2, ensuring membrane dryness, and eluting with Buffer AE.

The primer sequences used for detecting Mycobacteria RD4 and RD1 genes were as follows: RD4 forward: 5'-AAT GGT TTG GTC ATG ACG CCT TC-3', RD4 reverse: 5'-CCC GTA GCG TTA CTG AGA AAT

TGC-3' (176 bp amplicon); RD1 forward: 5'-CCC TTT CTC GTG TTT ATA CGT TTG A-3', RD1 reverse: 5'-GCC ATA TCG TCC GGA GCT T-3' (110 bp amplicon) (Taylor et al., 2007; Halse et al., 2011).

The duplex PCR followed the protocol outlined by Sonekar et al. (2021) with slight modifications. In the PCR reaction setup, components and concentrations were: 1X DreamTaq Green Buffer (1 µl), 200 µM dNTP mix (1 µl), 10 pm/µl Forward primer RD4 (0.5 µl), 10 pm μl^{-1} Reverse primer RD4 (0.5 µl), 10 pm μl^{-1} Forward primer RD1 (0.5 µl), 10 pm μl^{-1} Reverse primer RD1 (0.5 µl), Template DNA (2 µl), 3U μl^{-1} Taq Polymerase (0.2 µl), NF water (3.8 µl), resulting in a total reaction volume of 10 µl.

The PCR cycles included denaturation at 95°C for 7 minutes, subsequent cycles at 95°C for 1 minute, annealing at 59°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes over 35 cycles. Electrophoresis involved a 1.6 percent agarose gel in 1X TBE buffer with 0.5µg ml^{-1} ethidium bromide, run for 45 minutes at 70 volts. Gel documentation system visualization and photographic data recording concluded the process.

2.3. Identification of associated risk factor for occurrence of Mycobacteria spp. in small ruminants

For the risk factor analysis, a risk factor proforma was completed during the sample collection process by obtaining consent and verbally explaining to respondents the purpose of sample collection and questionnaire administration. A thorough statistical examination was carried out to assess the prevalence of *Mycobacteria* infections and their associated factors, employing a significance level of $p \leq 0.05$. Odds ratios (OR) with corresponding 95% confidence intervals (CI) were computed to measure the strength of these associations. The data were structured in Microsoft Excel and analyzed using SPSS version 25. Primary univariable analyses, encompassing $\chi 2$, Fisher's exact, and univariable logistic regression tests, were undertaken to explore associations and significance. The goal was to identify potential risk factors associated with *Mycobacteria* positivity, leading to subsequent multivariate logistic regression analysis.

3. RESULTS AND DISCUSSION

3.1. Molecular detection of Mycobacteria spp. from small ruminants

With a significant sample size of 795, the molecular detection through duplex PCR identified merely six positive samples, representing a modest 0.75% of the total samples (Figure 1). Among the goats sampled (n=756), 0.79% tested positive for *M. bovis*, with no positive cases

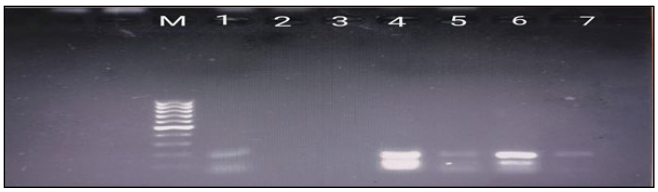

Figure 1: Duplex polymerase chain reaction (PCR) for detecting and differentiating *Mycobacterium* species. Lane M: 100 bp Marker, Lane 1: *M. bovis* Positive control (110 bp +176 bp), Lanes 4, 5, 6 and 7: *M. bovis* positive samples (110 bp +176 bp), Lane 2 and 3: Negative samples

for *M. tuberculosis*. In sheep (n=39), no positive cases were observed. Female goats (n=747) showed a prevalence of 0.67% for *M. bovis*, whereas male goats (n=48) had a higher prevalence of 2.08%. The prevalence varied across age groups, with the highest rate of 2.63% observed in 2.5-year-old goats (n=114). Blood samples (n=750) revealed a prevalence of 0.80% for *M. bovis*. Overall, the total prevalence of *Mycobacteria* spp. in the sampled small ruminants was 0.75%. Among the 131 small ruminant flocks, six tested positive, resulting in a herd prevalence of 2.6%.

3.2. Analysis of risk factor associated with BTB at animal level

In the univariable analysis investigating risk factors linked to *Mycobacteria* spp. prevalence at the animal level (Table 1), among the 795 animals, 6 goats tested positive, resulting in a prevalence of 0.79% (OR=0.992, 95% CI: 0.986-0.998, p=1.0). No positive cases were observed in sheep. Female goats exhibited a prevalence of 0.67% (OR=3.157, 95% CI: 0.362-27.576, p=0.313), and male goats had a higher prevalence of 2.08%. The prevalence varied across different age groups, with the highest prevalence of 2.63% observed in 2.5-year-old goats (OR=1.999, 95% CI: 0.907-4.404, p=0.086). Blood samples showed a prevalence of 0.80% (OR=0.000, 95% CI: NA, p=0.997). The significance level greater than 0.05 indicated that none of the factors were significantly associated with the occurrence of bovine tuberculosis at the animal level.

3.3. Analysis of risk factor associated with BTB at farm/flock level

The univariable analysis reveals significant associations with *Mycobacteria* spp. prevalence, with factors such as the number of small ruminants (OR=1.158, p=0.003) and introduction of new animals in the last 12 months (OR=1.090, p =0.034) showing notable impacts. Urban flock locations and absence of biosecurity measures also exhibit higher prevalence, though not statistically significant (Table 2).

Multivariable logistic regression investigation indicated that the variable "Number of small ruminants kept" is

Table 1: Univariable analysis showing risk factors associated with *Mycobacteria* spp. prevalence at animal level

Variable	Category	Total number of animal	No. of animal found positive	Prevalence (%)	p-value	OR	95%CI
Species	Goat	756	6	0.79	1.0	0.992	0.986-0.998
	Sheep	39	0	0			
Sex	Female	747	5	0.67	0.313	3.157	0.362-27.576
	Male	48	1	2.08			
Age (Year)	1	99	0	0	0.086	1.999	0.907-4.404
	1.5	44	0	0			
	2	365	1	0.27			
	2.5	114	3	2.63			
	3	171	2	1.17			
	4	2	0	0			
Type of sample	Blood	750	6	0.80	0.997	-	-
	Aborted foetus	33	0	0			
	Vaginal swab	9	0	0			
	Placental tissue	3	0	0			

p: Significance level (<0.05), OR: Odds ratio; CI: Confidence interval

Table 2: Univariable analysis showing risk factors associated with *Mycobacteria* spp. prevalence at Small ruminant farm/ Flock level

Variable	Category	Total no. of farm/flock	No. of positive farm/flock	Prevalence (%)	*p*-value	OR	95% CI
No. of small ruminant kept	<5	3	0	0	0.003	1.158	1.052-1.276
	5-10	22	0	0			
	>10	106	6	5.66%			
Flock location	Rural	111	4	3.60%	0.228	2.972	0.507-17.437
	Urban	20	2	10%			
New animal introduced in last 12 month	No	58	0	0	0.034	1.090	1.017-1.167
	Yes	73	6	8.22%			
Other species on farm	No	48	1	2.08%	0.414	3.013	0.341-26.581
	Yes	83	5	6.02%			
Vaccination	No	109	6	5.50%	0.589	0.945	0.903-0.989
	Yes	22	0	0			
Husbandry system	Extensive	58	2	3.45%	0.860	-	-
	Semi intensive	19	1	5.26%			
	Intensive	54	3	5.55%			
Quarantine of animal	No	90	6	6.67%	-	-	-
	Yes	41	0	0			
Floor spacing	Inadequate	41	2	4.88%	1.0	0.907	0.159-5.162
	Adequate	90	4	4.44%			

Table 2: Continue...

Variable	Category	Total no. of farm/flock	No. of positive farm/flock	Prevalence (%)	*p*-value	OR	95% CI
Presence of ticks	No	38	1	2.63%	0.672	2.102	0.237-18.618
	Yes	93	5	5.34%			
Biosecurity measures adopted	No	103	6	5.82%	0.340	0.942	0.898-0.988
	Yes	28	0	0			
History of reproductive disorder	No	92	6	6.52%	0.178	0.935	0.886-0.987
	Yes	39	0	0			
Proper disposal of placenta	No	34	0	0	0.338	1.066	1.013-1.122
	Yes	97	6	6.18%			
Mastitis	No	104	4	3.85%	0.603	2.0	0.346-11.544
	Yes	27	2	7.41%			
Whether bury dead animal	No	42	2	4.76%	1.0	0.941	0.165-5.354
	Yes	89	4	4.49%			

p: Significance level (<0.05), OR: Odds ratio; CI: Confidence interval

statistically significant (*p*=0.004), with an odds ratio (OR) of 1.155 (Table 3). This suggests that for each additional small ruminant, the odds of the outcome increase by approximately 15.5%. The 95% confidence interval for the odds ratio is between 1.046 and 1.274. The variable "New animal introduced in last 12 months (Yes)" does not contribute significantly with a p-value of 0.997. The number of small ruminants kept is a significant predictor of the outcome, while the introduction of new animals in the last 12 months does not provide meaningful information in this logistic regression model.

Tuberculosis remains a persistent concern in global sheep and goat farming, prompting extensive research on its prevalence and implications. Various studies, including those by Sonekar et al. (2021), Basit et al. (2015) and Zhang et al. (2013) utilized PCR methods to identify and assess the prevalence of *Mycobacterium* species across different animal species, emphasizing disease control and zoonotic risks. In the Chhattisgarh region, direct detection of bovine TB in blood samples revealed an overall prevalence of 0.75%. Comparisons with other studies, such as Tschopp et al. (2011) and Hena et al. (2012), showcased varied prevalence rates, emphasizing the multifaceted nature of the disease influenced by environmental conditions, management practices, and demographic factors. Al-Saqur et al. (2009) highlighted the increased risk of tuberculosis transmission to goats in close proximity to infected cattle, underscoring the zoonotic potential of tuberculosis. This comprehensive investigation into *Mycobacteria* spp. prevalence among different demographic groups provides crucial insights into the complexity of tuberculosis in small ruminants. The compilation of research on bovine tuberculosis (BTB) reveals multifaceted challenges at both individual animal and herd levels. Biffa et al. (2012) identified specific cattle breeds, particularly female and exotic breeds, as having a higher susceptibility to severe tuberculosis, while farming practices on a large scale and prolonged exposure to *M. bovis* increased the risk, especially in older animals. Mendez-Samperio et al. (2012) emphasized factors like co-infections, heredity, nutritional challenges, and helminth infections compromising immunity against *Mycobacterial* infections in cattle. Human risk factors associated with *Mycobacterial* infections include close contact between tuberculosis patients and cattle, food hygiene practices, and the public health implications of consuming contaminated milk and meat from infected cattle (Kader et al., 2023; Smita et al., 2021; Gompo et al., 2020).

In the context of small ruminants, studies by Tschopp et al. (2011), Katale et al. (2013), and Silaigwana et al. (2012) revealed variations in prevalence rates due to diverse management practices, sampling methods, and location-specific disease-control strategies. The proximity of goats to cattle, sharing water sites and pastures, contributed to a higher positivity rate in goats (Radostits et al., 2000). Zoonotic tuberculosis risk factors in humans were explored through questionnaire surveys, with Cook et al. (1996) and Ameni et al. (2002) establishing a significant link between human and animal tuberculosis reactors. Raw milk consumption emerged as a critical risk factor in many studies (Cordova et al., 2012; Bapat et al., 2017), and socio-economic status showed a highly significant association with tuberculosis infection (Ameni et al., 2002).

Table 3: Multivariable analyses of risk factors for *Mycobacteria* spp. infection at small ruminant farm/flock level

Variable	*p*-value	OR	95% CI
No. of small ruminant kept	0.004	1.155	1.046-1.274
New animal introduced in last 12 month	0.997	0.000	0.000
Constant	0.001	0.000	-

p: Significance level (<0.05), OR: Odds ratio, CI: Confidence interval

4. CONCLUSION

This two-year study in Chhattisgarh, India, focused on detecting Bovine Tuberculosis (BTB) in small ruminants and analyzing associated risk factors. It found a 0.75% individual animal-level and 2.6% herd-level prevalence of BTB. Herd-level risk factors included the number of small ruminants and introducing new animals. However, individual factors like species, age, sex, or sample type showed no significant associations. A significant predictor of BTB prevalence was the number of small ruminants kept, highlighting its role in herd-level transmission. The study underscores the need for ongoing research to develop effective control strategies for zoonotic tuberculosis in small ruminants.

5. FURTHER RESEARCH

The future work for this study could involve further investigations into the molecular epidemiology of *Mycobacterium* species in small ruminants, aiming to identify specific strains and their transmission patterns. Furthermore, research focusing on the development and evaluation of vaccination approaches and diagnostic tools tailored to small ruminants in the Chhattisgarh region could enhance disease management efforts. Addressing these aspects would contribute to a more nuanced understanding of bovine tuberculosis in small ruminants and aid in the formulation of effective control programs.

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