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CLINICO-PATHOLOGICAL AND BACTERIOLOGICAL STUDIES ON CASEOUS LYMPHADENITIS IN SMALL RUMINANTS

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Corynebacterium pseudotuberculosis is an intracellular bacterium, proliferates inside the macrophages, causes caseous lymphadenitis in small ruminants and is also responsible for human lymphadenitis. In current study, clinical signs and necropsy lesions suggestive of caseous lymphadenitis were observed in Chinkara deer (n=36), spotted deer (n=04) and Mouflen sheep (n=04) in a period of three years. Different pus samples were obtained from various superficial and internal lymph nodes, lungs, liver and spleen at the time of postmortem examination. All the collected samples were processed for microbiological investigations. *C. pseudotuberculosis* bacterium was cultured and identified in a total of 33 (75%) animals having clinical and necropsy lesions. The presence of *C. pseudotuberculosis* infection was significantly higher in older animals as compared to young animals. Morphologically, colonies were brown-yellowish in color, non-hemolytic on 5% sheep blood agar and biochemical characteristics were similar to *C. pseudotuberculosis* except urea test. Necropsy showed presence of different tubercles containing caseous material in visceral organs such as liver, spleen, lungs and intestinal mesenteric lymph nodes. Microscopically, tissue sections obtained from lungs, spleen, liver and intestinal mesenteric lymph nodes infected from *C. pseudotuberculosis* were plugged with abscess. The findings of our study indicate that wild animals are a reservoir for zoonotic Corynebacterium.

Keywords: Small ruminants, spotted deer, Mouflen sheep, caseous lymphadenitis, *C. pseudotuberculosis*, PCR

INTRODUCTION

Corynebacterium pseudotuberculosis is an intracellular, pleomorphic, facultative anaerobic, non-spore-forming, non-motile and is gram-positive bacterium (Dorella *et al.*, 2006; Alvarez *et al.*, 2017). The bacterial pathogen proliferates inside the macrophages and is known as an etiological agent of caseous lymphadenitis in small ruminants especially goats and sheep (Pavan *et al.,* 2012) and is also responsible for human lymphadenitis (Fontaine and Baird, 2010). It is well known that *C. pseudotuberculosis* poses a key virulence factor (phospholipase D) that favors the infectious agent to spread into blood vascular system by the distraction of cell membrane and inducing increased vascular permeability of vessels (Selvy *et al.,* 2011). *C. pseudotuberculosis* can infect different other species of animals such as cattle, camels, buffaloes horses and hedgehogs (Fontaine and Baird, 2007; Fontaine and Baird, 2010). Caseous lymphadenitis (CLA) predominantly occurs in several small ruminants and causes great economic loss in terms of poor milk production, compulsory surgical intervention, expensive and useless medication and decrease work activity of target animals (Kumar *et al.,* 2012; Chikhaoui andKhoudja, 2013; Aquino de Sa *et al.,* 2013). *C. pseudotuberculosis* infection causes huge losses particularly in different agro-ecological areas including tropical, subtropical, arid and semi-arid areas across Asia, Africa, South and Central America (Aquino de Sa *et al.,* 2013). High prevalence of *C. pseudotuberculosis* infection has been reported in different meat producing countries of the world such as United States, New Zealand, India, South Africa, Canada, Australia, Brazil, China and Iran(Dorella *et al.,* 2009; Kumar *et al.,* 2012; Zavoshti *et al.,* 2012; Alvarez*et al.*, 2017). *C. pseudotuberculosis* also causes an acute skin infection in buffaloes resulting poor milk production (Oedematus skin disease) in buffaloes (Selim, 2001; Mousa *et al.,* 2016). Several studies have reported that *C. pseudotuberculosis* causes enlargement of different lymph nodes including prescapular, submandibular, superficial, supramammary and prefemoral lymph. Initially these lymph nodes are filled with serous fluid which later on is converted to pus filled tubercles. The disease also causes lesions in other visceral organs including liver, lungs and

spleen (Zavoshti *et al.,* 2012). *C. pseudotuberculosis* biovar ovis induces diffused swellings in brisket regions, skin of fore limbs and hind quarter in exposed animals (Moussa *et al.,* 2016). The infectious agent is mainly resistant to low temperature and quickly enters into target animals via skin lesions. In human, *C. pseudotuberculosis* mainly results due to occupational exposure and infected milk. The visceral form of disease is normally reported in abattoirs workers (Silva *et al.,* 2013; Chikhaoui and Khoudja, 2013). Accurate diagnosis of caseous lymphadenitis due to *C. pseudotuberculosis* depends upon clinical signs, presence of tubercle having pus on different visceral tissues, reliable bacterial isolation, gene-based PCR assay, phenotypic features and biochemical tests infectious agent (Chikhaoui and Khoudja, 2013; Silva *et al.,* 2013). In published the literature no report is available about the presence of *C. pseudotuberculosis* infection in small ruminants in Pakistan. Therefore, in this study we report the presence of *C. pseudotuberculosis* infection depicting gross lesions in Chinkara deer, spotted deer and Mouflon sheep.

MATERIALS AND METHODS

***Pathology procedure*:** Necropsy of a total of 44 small ruminants including Chinkara deer (36), spotted deer (04) and Mouflon sheep (04) was carried out in a period of three years. All these cases were presented at University College of Veterinary and Animal Sciences, The Islamia University of Bahawalpur. The animals were examined carefully for presence of any external lesions. For internal lesions, necropsy of all animals was performed and the visceral organs were examined for presence of lesions. For histopathological analysis about 3 cm thick tissues were obtained from lungs, liver, spleen and lymph nodes. The collected samples were preserved immediately using 10% formaldehyde solution (Annas *et al*., 2015; Zubair *et al*., 2016). The collected tissues from different organs were processed by routine methods of dehydration in ascending grades of alcohol, clearing in xylene and embedding in paraffin wax (Ghaffar *et al.,* 2015). Finally, about 4 µm thick tissue sections were cut and stained with eosin and hematoxylin (Cho *et al*., 2015; Hasan *et al*., 2015) at Department of Pathology, University of Agriculture, Faisalabad.

***Isolation of pathogens and PCR application*:** For microbial investigation, different pus and tissue samples were collected in sterile tubes from all the infected animals. All the samples were processed at Department of Pathobiology, University College of Veterinary and Animal Sciences the Islamia University of Bahawalpur and Veterinary Research Institute, Zarrar Shaheed Road Lahore, Pakistan. For microbial culturing, all the collected samples were inoculated on blood agar supplemented with 5% sheep blood. The dry, whitish, convex and opaque colonies were obtained and were purified on cystinetellurite blood agar. For morphological features of infectious agent colonies were stained by using Gram staining procedures (Cabassi *et al*., 2015). All the inoculated purified microbial growth was subjected to different biochemical tests like nitrate reduction, urease and catalase. For confirmation, standard protocol of PCR was followed (Liu *et al*., 2016). *C. pseudotuberculosis,* PIP gene was amplified using previously used forward, 5′-ACTGCGGCTTTCTTTATTC-3′ and reverse 5′-GACAAGTGGGAACGGTATCT-3′ primer (Kumar *et al.,* 2012). For statistical investigations data obtained in current study was analyzed using Chi square analysis. Where appropriate the Odds ratio and 95% confidence interval was computed.

RESULTS AND DISCUSSION

Caseous lymphadenitis causes chronic infection in animals and is characterized by huge economic losses, contamination of meat, poor health and decrease wool production in small ruminants. In present study, we describe the brief clinical picture obtained by the attendants and pathological lesions in three species of small ruminants (Chinkara deer, spotted deer and Mouflon sheep) kept under tropical conditions. Different clinical signs such as dyspnea, lack of enthusiasm for feeding and drinking and keeping head downward were reported by the attendants. In spite of antibiotics (tribrissen 2-3 ml, gentamycin=1–1.5 ml, enrofloxacin = 0.4 ml), vitamins (B-complex), steroid and electrolytes therapy death occurred in animals. In present study, the results showed no significant differences in prevalence of *C. pseudotuberculosis* infection (Table 1) on the bases of sex of the animals (OR = 1.14, reciprocal = 0.88). The prevalence of *C. pseudotuberculosis* was significantly increased in old animals as compared to young animals ($P<0.002$) suggesting chronic nature of infection. Previously different studies showed that the prevalence of *C. pseudotuberculosis* increases with increase in age of animals (Arsenault *et al.,* 2003; Chikhaoui and Khoudja, 2013). No significant results were found on the basis of distribution of *C. pseudotuberculosis* among different species (Mantel-Haenszel Chi-sq = $P>1.00$). The frequency of different pathological lesions has been presented in Table 2. The infected animals without any external lesions like skin injuries, swelling of cervical, submandibular and prescapular lymph nodes showed small tubercles packed with caseous materials in visceral organs including lungs, liver, spleen and mesenteric lymph nodes. The presence of caseous material in liver (75.00%) was the consistent findings in infected animals. Different other pathological lesions such as abscess in submandibular lymph nodes 23(52.27%), abscess in cervical lymph nodes 25(56.81%), abscess in lymph nodes of brisket region 17(38.63%), caseous materials in lungs 29(65.90%), caseous materials in spleen 27(61.36) and

Table 1. PCR based distribution of *C. pseudotuberculosis*infection in small ruminants

Sex/age	Animal examined	PCR positive		95% CI	Odd Ratio/ P value
		n	%		
Sex					
Female	29	22	75.86	57.99- 88.78	OR = 1.14 [reciprocal = 0.88]
Male	15	11	73.23	47.47 -90.90	
Species					
Chinkara deer	36	27	75.00	59.05- 87.06	Mantel-Haenszel Chi-sq P>1.00
Spotted deer	4	3	75.00	24.23- 98.75	
Mouflon sheep	4	3	75.00	24.23- 98.75	
Overall	44	33	75.00	60.71- 86.09	-
Age groups (Years)					
1-2	8	2	25.00	4.43- 61.17	Mantel-Haenszel Chi-sq P<0.002
3-4	19	16	84.21	62.79- 95.82	
5-8	17	15	88.23	66.27- 97.98	

Table 2. Overall frequency of different lesions examined in animals (n=44) suggestive of *C. pseudotuberculosis* infection.

Pathological lesions	Frequency		95% CI
	n	%	
Abscess in submandibular lymph nodes	23	52.27	37.6-66.65
Abscess in cervical lymph nodes	25	56.81	41.98-70.79
Abscess in lymph nodes of brisket region	17	38.63	25.19-53.54
Caseous material in liver	33	75.00	60.71-86.09
Caseous materials in lungs	29	65.90	51.07-78.71
Caseous materials in spleen	27	61.36	46.46-74.81
Caseous materials in internal lymph nodes	19	43.18	29.21-58.02

caseous materials in internal lymph nodes 19(43.18%) were observed in infected cases during postmortem examination. The visceral organs particularly lungs (Fig. 1), liver (Fig. 2), spleen (Fig. 3) and mesenteric lymph nodes (Fig. 4) were consolidated and exhibited small tubercular lesions having pasty and odorless pus.

Figure 1. Liver tissue showing small tubercles containing caseous material suggestive of infected with *Corynebacterium pseudotuberculosis.*

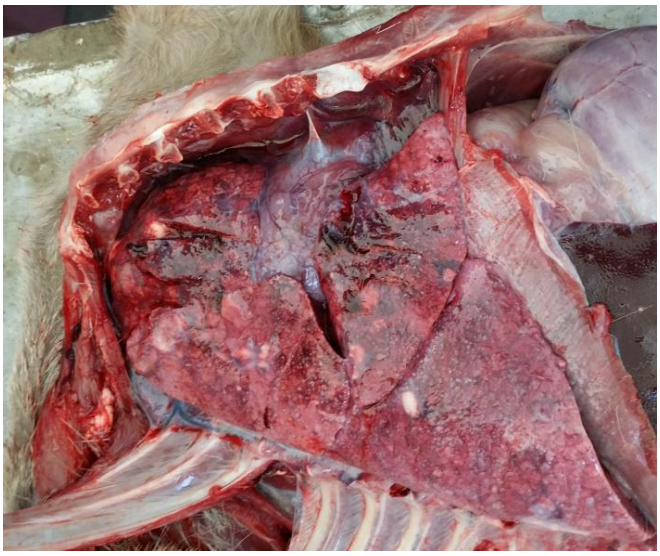

Figure 2. Lungs showing small tubercles containing caseous material suggestive of infected with *C.pseudotuberculosis.*

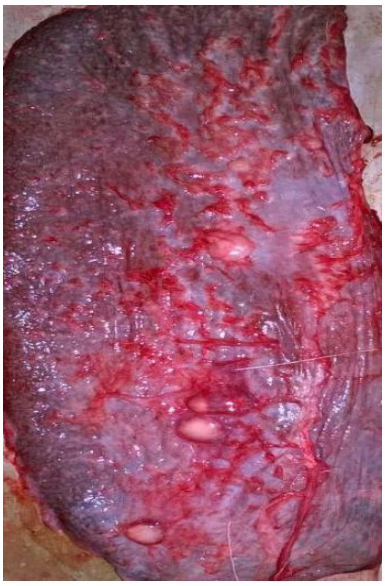

Figure 3. Spleen showing small tubercles of variable size containing caseous material.

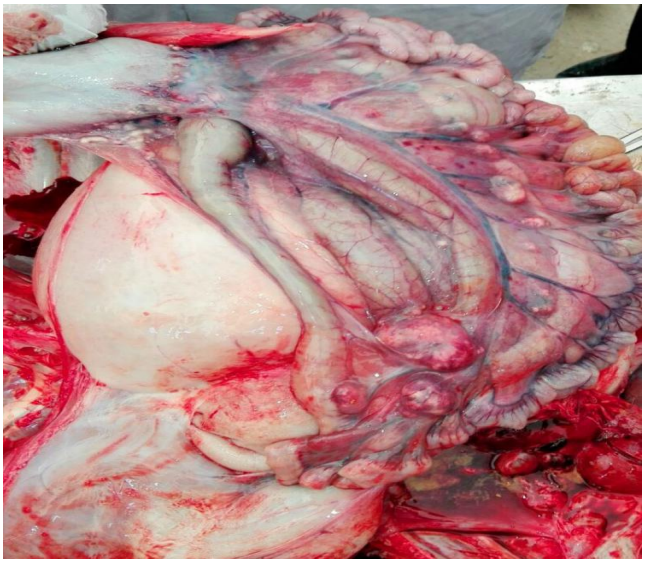

Figure 4. Intestinal mesenteric lymph nodes containing caseous and purulent exudates.

The mesenteric lymph nodes were swollen and had caseous material. The cut surfaces of all the visceral organs showed multiple abscesses frequently distributed within small tubercles. Abscess formation in cut sections of lungs and liver due to *C. pseudotuberculosis* in alpaca has been reported (Braga *et al.,* 2006; Fontaine and Baird, 2007). Previous studies have disclosed that presence of small tubercles packed with caseous material in lungs, liver, internal lymph nodes and mesenteric lymph nodes are the characteristic features of visceral forms of caseous lymphadenitis caused due to *C. pseudotuberculosis* (Yeruham *et al.,* 2003; Zavoshti *et al.,* 2012; Chikhaoui and Khoudja, 2013). Different studies have also reported similar pathological lesions in infected animals (Pratt *et al.,* 2005; Quino de Sa *et al.,* 2013; Chikhaoui and Khoudja, 2013). *C. pseudotuberculosis* also causes liver abscess, skin and lymph nodes lesions in human, sheep, alpaca and buffaloes (Paton *et al.,* 2003; Guimarães *et al.,* 2011; Moussa *et al.,* 2016). Previously abscess formation in 80–87% of internal and external lymph of sheep, alpacas and prescapular and scapular lymph nodes of infected buffaloes due to *C. pseudotuberculosis*has been reported (Anderson *et al.,* 2004; Moussa *et al.*, 2016). Histopathological investigations of infected tissues from visceral organs liver, spleen, and lungs showed chronic inflammatory response comprising of mononuclear cell infiltrations and connective tissue proliferations. The infected lung tissues also showed eosinophilic and proteinous materials. Morphologically the bacterial pathogens were identified on the basis of growth characteristics brown-yellowish in color and non-hemolytic colonies on blood agar after 48 h of incubation. Microscopically the pathogens appeared as Gram positive through Gram staining. Biochemically the infectious agents showed positive reactions to nitrate reduction, urease and catalase tests (Kumar *et al.,* 2012). *C. pseudotuberculosis* bacterium was confirmed by amplification of PIP gene (Fig. 5) in a total of 33 isolated genomic DNA from gram positive culture. Similar reports are also available in published literature (Kumar *et al.,* 2012; Aquino de Sa *et al.,* 2013). The findings of our study indicate that wild animals are a reservoir for zoonotic *C. pseudotuberculosis* infection.

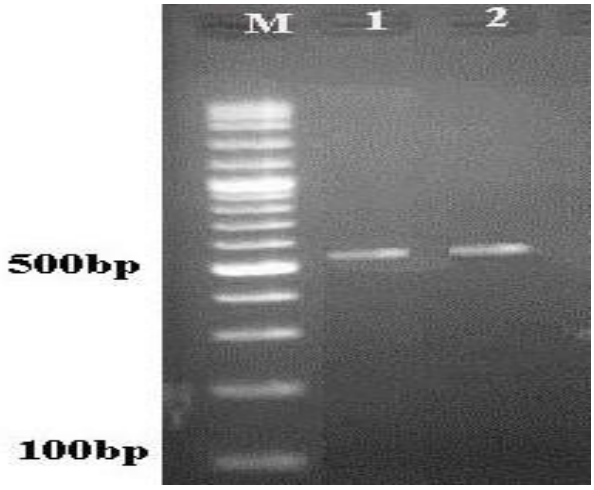

Figure 5. PCR amplification of PIP gene 551-bp fragment of *C. pseudotuberculosis.* Lane M, DNA marker (100 bp) and lanes 1–2 positive samples of PIP gene.

Conclusions: The results of current study confirmed the presence of C. pseudotuberculosis infection in small

ruminants which can be valuable tools for the early diagnosis. Furthermore, the findings of this study are best contribution for epidemiological surveillance in small ruminants.

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