Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection among ruminants in Nandanakanan Zoological Park(NZP), Odisha

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IN
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By

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CERTIFICATE-I

This is to certify that the thesis entitled **"Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection among ruminants in Nandanakanan Zoological Park(NZP), Odisha"** submitted in partial fulfilment of the requirements for the award of thedegree of **Master of Veterinary Science (Veterinary Epidemiology and Preventive Medicine)** to the Orissa University of Agriculture and Technology is a faithful recordof bonafide and original research work carried out by **Priyabrata Mohanty** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help received by him from various sources during the course of investigation has been duly acknowledged.

CHAIRMAN
ADVISORY COMMITTEE

CERTIFICATE-II

This is to certify that the thesis entitled **"Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection among ruminants in Nandanakanan Zoological Park(NZP), Odisha"** submitted by **Priyabrata Mohanty** to the Orissa University ofAgriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the degree of **Master of Veterinary Science (Veterinary Epidemiology and Preventive Medicine)** has been approved/disapproved by the students'advisory committee and the external examiner.

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Bhubaneswar
Date: **(Priyabrata Mohanty)**

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ABBREVIATIONS USED

JD	: Johne's Disease
MAP	: *Mycobacterium avium* subspecies *paratuberculosis*
CD	: Crohn's Disease
UC	: Ulcerative Colitis
IBD	: Inflammatory Bowel disease
ZN staining	: Ziehl–Neelsen staining
ELISA	: Enzyme Linked Immunosorbent Assay
PCR	: Polymerase Chain Reaction
Ns PPA	: Native Semi-Purified Protoplasmic Antigens
OD	: Optical Density
DNA	: Deoxyribo Nucleic Acid
TRIS HCL	: tris(hydroxymethyl)aminomethane hydrochloric acid
WBC	: White Blood Corpuscles
Na_2EDTA	: Sodium Ehylene DiamineTetra acetic Acid
PBS	: Phosphate Buffer Saline
mg	: Miligram
ml	: Mililitre
SDS	: Sodium Dodecyl Sulphate
CTAB	: Cetrimonium bromide
BSA	: Bovine Seum Albumin
µg	: micro gram
TE buffer	: Tris-EDTA buffer
nm	: nanometer
dNTP	: Deoxyribo Nucleotide TriPhosphate

HPLC water : High Performance Liquid Chromatography

PBST : Phosphate buffered saline with tween 20

ABSTRACT

A study was undertaken on Paratuberculosis infection in **Nandanakanan Zoological Park(NZP), Odisha** from January 2018 to June 2018 to assess the current status of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection among ruminants in Nandanakanan Zoological Park(NZP). Screening of 17 faecal samples by ZN staining revealed 3 positive sample with +1 rating. These 3 sample were subjected to PCR reaction 25 serum were subjected to indigenous i_ELISA reaction which revealed 4 sample positive. Blood of these 4 positive samples and another 9 negative samples in i_ELISA kit were subjected to PCR. Out of 13 samples subjected to PCR reaction revealed all samples negative for *Mycobacterium avium* subspecies *paratuberculosis.* Detection of MAP by IS900 PCR in faecal samples was rapid but low thorough-put as MAP sheeding at early and subclinical stage is low and intermittent. From the result of ZN staining and i_ ELISA, it is evident that subclinical infection of MAP is present in the herd of ruminant of Nandanakanan Zoological Park(NZP).PCR may not detect MAP in sub-clinical cases. Also low prevalence may be due to chemoprophylactic administration of antitubercular drug i.e. Mycocox-E for 3 months in a year. All negative samples found in single PCR may be subjected to culture of bacterium from faecal sample for confirmation of MAP status. However, in ZN staining technique prevalence of MAP was 17.65% i.e in Hog deer, White buck and Chousinga. The sero-prevalence of MAP was detected for about 19.2% in spotted deer by indigenous ELISA.

CHAPTER-I

INTRODUCTION

Paratuberculosis (Johne's disease) is a chronic infectious disease affecting wild and domestic ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease is prevalent worldwide and has a significant financial impact on national economy (Losinger *et al*., 2006). It has recently emerged as one of the most wide-spread, highly prevalent and economically devastating infectious diseases of domestic livestock around the world (Sohal *et al*., 2015).

Mycobacteria belong to the phylum Actinobacteria and are characterized by rod shape, acid-alcohol fastness (distinguishable by Ziehl-Neelsen staining), high genomic content of guanine and cytosine (61-71%) and the presence of long and complex mycolic acids in the cell wall (Shinnick and Good, 1994). MAP is a gram-positive, andextremely slow growing bacillus (Lilenbaum *et al*., 2007). *Mycobacterium avium* subspecies *paratuberculosis*is an intracellular pathogen that replicates in macrophages in the lamina propria and is shed into the faeces during disease.

Thick, waxy and lipid-rich cell wall, give it a general survival advantage and increased resistance to high temperatures (Grant *et al*., 2005), low pH, salt and chemicals, such as chlorine (Rowe and Grant, 2006; Donaghy *et al*., 2004). Although it has been claimed that proper pasteurisation should kill all MAP (Rademaker *et al*., 2007 and Stabel, 1997), viable MAP has been reported in retail milk (Ayele *et al*., 2005; Ellingson *et al*., 2005a; Grant *et al*., 2002a). MAP has been shown to be able to survive for long periods of time in the environment, complicating the eradication process of the disease (Whittington *et al*., 2005). Probably, the thickness of the cell wall is also contributing to its slow growth, due to restricted uptake of nutrients, although MAP's inability to produce mycobactin is thought to be one of the main constraints.It has also been shown that MAP can survive in protozoa, insects and biofilms in the environment and has been reported in rivers and catchment areas (Rowe and Grant, 2006; Pickup *et al*., 2005; Whan *et al*., 2005). Among wild ruminants of India disease prevalence varies from 15 -61.8 % basing on the method of diagnosis and species of wild ruminant (Singh *et al*.,2016).

Ana *et al.* (2017) reported the prevalence of paratuberculosis in free ranging red deer (*Cervus elaphus*). Corn *et al.* (2005) isolated MAP from various wild monogastric mammals and birds, such as raccoons, opossums and starlings. MAP has also been isolated from a wide range of wild non-ruminant species (e.g. foxes, stoats, weasels, crows, hares, rabbits; Beard *et al.,* 2001;Greig *et al.,* 2003; Deutz *et al.,* 2005; Corn *et al.,* 2005; Judge *et al.,* 2005). Paratuberculosis is principally a disease of ruminants but disease is also noticed in nonruminant animal species (pigs, dogs, horses, cats, etc.) including free ranging animals (Blue bull, deer, rabbits) and primates (Castellano *et al*.,2012).Itis also reported in bighorn sheep (*Ovis canadensis*), bison (*Bison bison*), elk (*Cervus canadensis*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and reindeer (*Rangifer tarandus tarandus*) (Jessup *et al*., 1981; Buergelt *et al*., 2000).

Infected animals can shed MAP organisms in their faeces for months to years before they show clinical signs (Fecteau *et al*., 2013). The risk of transmission of paratuberculosis from wild animals to domestic livestock appears to be real and should not be neglected (Daniels *et al*., 2003). Faeco-oral transmission of MAP is the most common way for the disease to spread among cattle (Stabel, 1997; Chiodini *et al*., 1984). The animals are most susceptible at the age below 30 days and usually ingest the bacteria when suckling from an infected dam that sheds MAP directly into her milk and colostrum (Streeter *et al*., 1995; Sweeney *et al*., 1992). Heavily infected dams may also convey the organisms to foetuses *in utero* (Sweeney, 1996; Lawrence, 1956). The possible transmission of pathogens among wild species, domestic animals is of great concern when a disease is present in a wild host (Gortazar *et al.,* 2007). Wild ruminants may act as a reservoir and contribute to the spread of MAP through faecal shedding (Manning, 2001).

Paratuberculosis in animals is a chronic wasting and debilitating disease characterized by weight loss, profuse diarrhoea, chronic progressive enteritis, protein loss, dehydration, wasting, and inevitably death. Body-condition indices can vary substantially depending on season, age, and reproductive status (Chan-McLeod *et al*., 1995). Adult red deer are infected, but only young animals (8–15 months old) may show a sudden onset of clinical signs, with rapid worsening of symptoms, weight loss and frequent diarrhoea. Adults rarely develop clinical signs, although they may be sero-

positive and/or show pathological lesions only on postmortem examination (Mackintosh *et al.,* 2004). It has been known to occur in outbreak form in young deer and elk and to progress more rapidly in these species than in cattle (Manning *et al*.,2001). Clinical form of the disease is also reported in several wild ruminant species (Taya et.al.,2012).

It is important to diagnose the disease as early as possible to reduce losses and also spread of infection to susceptible animals and humans. The diagnostic tests used to detect infection with MAP can be categorized into those that identify the organism and those that identify an immunological reaction to the organism.

Direct diagnosis of paratuberculosis involves culture of MAP from faeces. Culture of visible colonies can take more than 16 weeks (Collins, 1996) and practically all strains require supplement of mycobactin (Cocito *et al*., 1994). Mycobactin is a so called siderophore, an iron-chelating agent that most other mycobacteria produce endogenously to assist the uptake of iron, thereby circumventing the active removal of intracellular iron by MAP-infected macrophages (Harris and Barletta, 2001). However, faecal culture is time consuming and expensive. Sample storage conditions can affect MAP viability and the subsequent ability to culture (Khare *et al*., 2008). The number of bacteria shed by subclinically infected animals may be below the threshold of detection in PCR reaction (Whittington and Sergeant, 2001).

Diagnosis mainly depends on the detection of acid fast bacilli by animals excreted in its faeces and immune response mounted by it. Shedding of MAP bacilli can be detected by Ziehl-Neelsen staining of faecal smears and by isolation of MAP DNA from faecal samples and subsequent amplification by IS900 PCR (Singh *et al*., 2013).

The only effective control measures are farm management strategies aimed at the reduction of MAP transmission and the culling of infected animal, which requires an accurate diagnostic test for detection (Sweeny *et al*., 1996). Poor sensitivity and specificity of current diagnostic tests have been major obstacles for the control of paratuberculosis in wild ruminant since disease gets transmitted prior to the development of clinical signs (Sohal *et al*.,2007 and Chaubey *et al*.,2016),

Prevention and control of Johne's disease is severely hindered due to its long incubation period, presence of undetected subclinical cases, absence of rapid MAP specific diagnostic tools and efficacious vaccines and lack of knowledge of strain diversity (Whitlock *et al*., 1996 and Stabel *et al*., 2004).

Mycobacterium avium subspecies *paratuberculosis* has been implicated in the etiology of Crohn's disease in humans (Bull *et al*., 2003). Interest in the disease is growing, not only because of the heavy economic loss for the farmers but also because of the involvement of MAP in the etiology of Crohn's disease (Hermon,2001).

Keeping the spectrum of host and public health significance of Johne's disease in view, a study on "**Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection among ruminants in Nandanakanan Zoological Park (NZP), Odisha."** is undertaken with following objectives :

- Identification of *Mycobacterium avium* subspecies *paratuberculosis* in captive ruminants of Nandankanan Zoological Park by faecal examination through staining, culture and molecular test.
- Bio-detection of antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in captive ruminants of Nandankanan Zoological Park using indirect ELISA (Indigenous kit).

CHAPTER-II

REVIEW OF LITERATURE

2.1 Epidemiology

2.1.1 Global

Soltys *et al*. (1967) found MAP in Moose (*Alces alces*) of USA .Temple *et al*. (1979) diagnosed paratuberculosis in a herd of 62 fallow deer (*Dama dama*) and 38 sika deer (*Cervus nipon*) maintained in an animal park in Ohio. A comparison was made of delayed-type hypersensitivity responses, results of in vitro lymphocyte immunostimulation tests, and isolation of Mycobacterium paratuberculosis on faecal culture in 19 deer.

Riemann *et al*. (1979), Temple *et al*. (1979) reported that 89 faecal samples from 52 axis deer (*Axis axis*) and 37 fallow deer (*Dama dama*), 5 (9.6%) and 3 (8.1%), respectively, contained MAP in at Point Reyes National Seashore, California, USA . Culture of intestinal necropsy samples from the same deer indicated that 3 (5.8%) of the axis deer and 2 (5.4%) of the fallow deer were infected with MAP. Jessup *et al*. (1981) found MAP in Tule elk (*Cervus elaphus nannodes*) in USA.

Chiodini and Van Kruiningen (1983) states that White-tailed deer (*Odocoileus virginianus*) act as reservoir for MAP Ten white-tailed deer were shot and killed at a Connecticut farm that had a 6-year history of bovine paratuberculosis, and organs from these animals were examined for evidence of paratuberculosis. Mycobacterium paratuberculosis was isolated from the caecal lymph node, terminal ileum, and ileocaecal valve of 1 deer and from the caecal lymph node of another. Characteristic lesions and acid-fast bacilli were not observed. It was concluded that infected deer could serve as sources of infection for domestic stock.

De Meurichy *et al*. (1985) reported MAP in Pudu (*Pudu pudu*) of Belgium.

Momotani *et al.* (1988) stated that the MAP infect the M cells of the follicle in intestinal epithelium and then engulfed by intestinal macrophages leads to replication and viable for several months to years and development of disease.

Von Weber and Gurke (1992), Von Weber *et al*. (1992) reported MAP in fallow deer of Germany (Power *et al*., 1993) observed MAP in European red deer (*Cervus elaphus*) of Ireland .De Lisle *et al*. (1993) observed MAP in European red deer (*Cervus elaphus*) of New Zealand .Fawcett *et al*. (1995) observed MAP in European red deer (*Cervus elaphus*) of Scotland. Rohonczy *et al*. (1996) found MAP in Elk (*Cervus elaphus*) of Canada.

Reports of National Animal Health Monitoring System(1997) reveal an increased sero-prevalence of Johne's Disease, world wide ranging from 15% to 78%, 68.1% of US dairy farms are infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and also suggests that at least one out of every four United States dairy may have a relatively high per-centage of Johne's-infected cows in their herds.

Manning *et al.* (1998) stated that Elk (*Cervus elaphus*) at Point Reyes National Seashore in California and a population of bighorn sheep (*Ovis canadensis*) in Colorado have remained infected over several years. Manning *et al.* (1998) found MAP in Elk (*Cervus elaphus)* of USA .

Greig *et al.* (1999) stated that although the frequency of transmission from wildlife to domestic animals has not been documented, several reports suggest that infection may be spread from domestic animals to wildlife. Pavlik *et al*. (2000) observed MAP in European red deer (*Cervus elaphus*) of Czech Republic.

Nebbia *et al.* (2000) suggested the involvement of roe deer (*Capreolus capreolus*) in the epidemiology of *Mycobacterium avium* subspecies *paratuberculosis* in isolated areas with associated livestock infection.

Nebbia *et al.* (2000) identified paratuberculosis in the United States-V hunting district, and clinical cases had been found in red deer .Nether-lands recorded 55% prevalence (Muskens *et al*.,2000) and in Denmark re-ported 47% prevalence of JD (Nielson *et al*.,2000).

Pavlik*et al*. (2000) monitored the occurrence of paratuberculosis in seven species of wild ruminants over an eight-year period. A total of 6935 animals of wild ruminant species were involved: 1692 red deer (*Cervus elaphus*), 1534 roe deer (*Capreolus capreolus*), 1905 fallow deer (*Dama dama*), 125 sika deer (*Cervus nippon*), 1518 mouflons (*Ovis musimon*), 27 bezoars (*Capra aegagrus*), and 134 chamois (*Rupicapra rupicapra*). Paratuberculosis was diagnosed in faeces of 288 animals (4.2% of examined animals). The most infected were red deer (n=206), which are considered more susceptible to this disease than cattle.

Manning *et al.* (2001) reported that up to one-third of zoos accredited by the American Zoo and Aquarium Association have reported at least one culture-confirmed case of paratuberculosis since 1995.

Studies undertaken in the last few decades showed that paratuberculosis is worldwide in distribution and highly endemic in the dairy cattle herds of the developed countries (Kennedy *et al*., 2001 and Ayele *et al*.,2001).

Ignasi *et al.* (2002) reported paratuberculosis in a population of approximately 1,000 free-ranging fallow deer (*Dama dama*) sampled from 1997–98 in the Regional Hunting Reserve of El Sueve (Asturias, Spain). Five of eight animals observed with diarrhoea were diagnosed as having paratuberculosis on the basis of gross lesions at postmortem examination and histopathology. In two deer,

Mycobacterium avium subspecies *paratuberculosis* was cultured and identified by polymerase chain reaction. Indirect enzyme-linked immunosorbent assay and immunodiffusion tests were used to evaluate sera from 33 adult deer from this population. All fallow deer tested were seronegative.

Daniels *et al* (2003) observed that the existence and importance of wildlife reservoirs of MAP in the transmission cycle are still undetermined, and few investigations have examined the role of wildlife in the epidemiology of this important disease.

Cousens*et al.* (2004) reported that paratuberculosis is not only chronic but is also currently incurable and often remains in a subclinical state for years.

Murray *et al.* (2008) compares the results and suitability of serological testing, microscopic examination, deoxyribonucleic acid (DNA) detection, and bacterial culture for detecting *Mycobacterium avium* subspecies *paratuberculosis*(MAP*)* infection in asymptomatic farmed white-tailed deer (WTD) (*Odocoileus virginianus)*.Behr *et al.* (2008) found MAP as the cause of Crohn's disease (CD) in human beings.

Robino *et al.* (2008) examined Seventy-seven red deer (*Cervus elaphus hippelaphus*), 40 roe deer (*Capreolus capreolus*) from the Northwestern (NW) Alps (Turin Province, NW Italy) and 29 roe deer from the NWA pennines (Alessandria province, NW Italy) for the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) by culture, IS900 nested polymerase chain reaction (PCR) and IS1311 PCR restriction endonuclease analysis for strain characterisation. MAP identification (nested PCR and/or culture) allowed us to detect 32.9% MAP-infected red deer and 22.5% infected roe deer in the NW Alps and 41.4% MAP infected roe deer in the NWA pennines. On the basis of the polymorphism present in the IS1311 sequence, all MAP isolates were characterised as cattle strains.

Bastida *et al.* (2011) stated that paratuberculosis was first described in Germany in 1895 by Johne and Frothingham.

Taya *et al.* (2012) collected 561 faecal samples between 2007 and 2009 we from caribou (*Rangifer tarandus*) representing 10 herds of migratory caribou, two herds of caribou from Greenland, and three populations of boreal woodland caribou. Faeces were tested for MAP by bacterial culture and PCR targeting the IS900 insertion sequence. In total, 31 samples from eight different populations representing all three ecotypes were found positive for MAP by PCR, with one sample from the Riviere-aux-Feuilles herd also being culture positive for the type II (cattle) strain.

Sarno *et al.* (2013) collected total 198 faecal samples (69 red deer (*Cervus elaphus*), 51 roe deer (*Capreolus capreolus*), 51 chamois (*Rupicapra rupicapra*), and 9 ibex (*Capra ibex*)). The majority of the hunted animals were on average 2 years old and originated from the central and eastern part of Switzerland. Using the described methods, none of the pooled faecal samples from the wild ruminants were found to be positive for viable MAP.

Ana *et al.* (2017) collected sample from 877 free ranging red deer legally hunted in the Centre-eastern Portugal. Thirty-seven (4.2%) kidneys revealed acid-fast bacilli when screened with the Ziehl-Neelsen technique. MAPwas detected by IS*900* polymerase chain reaction (PCR) in thirty (81.1%) of the Ziehl-Neelsen positive kidneys. Subsequent PCR and/or culture from the different organs of the 37 examined animals allowed to detect 86.4% (32 animals) infected red deer.

2.1.2 India

Twort *et al.* (1913) reported that in India, the first case of paratuberculosis was observed in Lahore (undivided India) in 1913, followed by another case in 1918 from a Military dairy farm (Sheather *et al*.,1918). Thereafter (1918 to 1990), the disease was investigated on a limited scale in the country. It may be due to a lack of

indigenous and cost-effective diagnostic kits and methodologies and ill-equipped infrastructure of laboratories and trained workforce in the country; consequently, variable prevalence has been reported from different parts of the country (Kulshrestha, 1980).

Muskens *et al.* (2000) reported that JD was first diagnosed in India in 1913 at Hissar followed by reports from different parts of the country with incidence ranging from 1.78% to 1.9%.

Singh *et al.* (2000) reported sero-prevalence of 31.9% and 23.3% in Uttar Pradesh and Punjab states respectively. The apparent prevalence of JD is 13.39% and 16.26% in Gujarat and Andhra Pradesh respectively (Lall *et al*., 1963). Approximately 29.0% (28.6% in buffalo and 29.8% in cattle) of sero-prevalence of JD is reported in northern India.

Barbaruah and Joseph (2008) studied that the morbidity rates are very high and distributed over a period of time thus losses in production go unnoticed and have never been estimated in India despite low per animal productivity.

Singh *et al.* (2008) conducted several studies in India reporting MAP in different livestock species, animal derived food and food products, natural resources and human beings.

Kumar *et al.* (2010) found 10 sample positive from culture of 42 faecal sample of Blue bulls (*Boselaphus tragocamelus*).

Singh *et al.* (2010) found 20 percent positive in microscopy and 15 percent positive in culture for MAP from faecal sample of Hog deer (*Axis porcinus*).

Singh *et al.* (2011) reported 46.8 percent positive in microscopy and 61.8 percent positive in culture for MAP from faecal sample of Wild bison (*Bos gaurus*).

2.1.3 Odisha

Rahman *et al. (1985*) reported 7.4% prevalence in ZN staining in sheep.

Biswal *et al.* (2018) collected 22 serum samples were collected from goats in and around Bhubaneswar, Odisha and subjected to indirect ELISA by indigenous ELISA (i_ELISA) kit. An apparent prevalence 68.19% (15/22) was recorded by i_ELISA where the S/P ratio of the positive control was considered to be 1(100%).

2.1.4 Epidemiology in Human

Venugopalen *et al.* (1980) suggested that in the last ten years, However,, Crohn's disease has been reported more frequently from different parts of India, especially the south. Mishina *et al.* (1996) cultured viable MAP from potable chlorinated municipal water in USA.

Selby *et al.* (2000) stated that Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are complex disorders reflected by wide variations in clinical practice. IBD has long been considered to affect the populations of North America and Europe more than those of Asia. Crohn's disease was considered almost non-existent in India until 1986.

Gwozdz *et al*. (2000) reported presence of MAP in blood from patients with Crohn's disease should not be surprising since in animals with Johne's disease , MAP infection is systemic.

Naser *et al.* (2000) studied on MAP bacteremia and cultured MAP from the blood of 14 (50.0%) patients with Crohn's disease and two (22.0%) with ulcerative

colitis. Greenstein *et al.* (2004) stated that these may be potential sources of MAP, if it is eventually accepted that MAP is zoonotic.

Naser *et al.* (2000) cultured MAP from the milk of two women with active Crohn's disease. Hermon *et al.* (2000) reportedhigh proportionsof MAP by using improved cultureand IS900 PCR. Grant *et al.* (2002) cultured viable MAP from commercial pasteurized milk in UK.

Sood *et al.* (2003) reported very high prevalence of ulcerative colitis from Northern India.

2.2 Clinical signs

Poddoubski (1957) observed that clinical signs of Johne's disease can be subtle, with weight loss being the primary symptom in *Rangifer tarandus*. Despite removing major parts of intestines in CD patients, the disease has been found to relapse (Ambrose *et al*., 1985)

Gilmour and Nyange (1989) observed that Roe deer are also exposed to MAP and become infected, but roe deer seem to have a low degree of susceptibility to paratuberculosis and do not usually develop clinical signs, which is contrary to what has been reported in red deer.

Chan-McLeod *et al*. (1995) studied that body-condition indices can vary substantially depending on season, age, and reproductive status.Collins *et al*. (1997) found that MAP infection either in animals or in human beings is incurable.

Hasonova *et al.* (2006) reported that clinical condition is characterized by poor body condition, progressive weight loss with or without diarrhoea, debility and emaciation. Production losses result from reduced milk yield, altered milk constituents, high somatic cell counts, poor feed conversion rate, increased

susceptibility to mastitis, reduced reproductive efficiency, premature culling and reduced slaughter weight and carcass quality.

Verma *et al.* (2013) suggested that MAP may persist in the intestines and other tissues for years without causing clinical disease. If stressed (nutritional, pregnancy, parturition,lactation, environmental or any other concurrent disease), subclinical infection develops into clinical disease. These animals continue to excrete bacilli in their faeces and milk on a regular basis (Garg *et al*.,2015).

2.3 Modes of Transmission

Lepper *et al*. (1989) found that after infection has taken place but before clinical symptoms are apparent, the infected animal likely sheds bacteria.

Manning *et al*. (1998) reported that the pathogenesis of MAP infection in caribou could be similar to that seen in other cervids, which progress more quickly from silent to subclinical infection than domestic cattle or sheep.

Whittington and Sergeant (2001) stated bovine calves as young as 6 months of age and domestic sheep as young as 12 months can shed MAP, although it is exceptional for shedding to occur this early in these species.

Whittington and Sergeant, (2001) suggested that *Mycobacterium avium* subspecies *paratuberculosis* can survive for several months in manure at cool temperatures but cannot replicate outside of a host.

Whittington *et al.* (2005) and Windsor (2003) reported that *Mycobacterium avium* subspecies *paratuberculosis* is primarily transmitted by the faecal–oral route, although intrauterine infection may also play a role.

Corn *et al*. (2005) suggested that MAP transmission may occur from wildlife to wildlife, wildlife to livestock and/or livestock to wildlife. Wild ruminants, as possible reservoirs for MAP, play an important role in paratuberculosis control/eradication programmes in domestic ruminants, as they may possibly affect the success of such programmes.

Ellingson *et al.* (2005) reported that since MAP escapes pasteurization temperature, milk of the infected animals is the most common source of transmission of MAP from animals to human beings.

Manning *et al*. (2001) observed that wild ruminants may act as a reservoir and contribute to the spread of MAP through faecal shedding.

Eisenberg *et al.* (2010) reported that MAP is excreted in faeces, milk and semen of subclinical or clinically infected animals resulting in increased environmental contamination. Newborn animals acquire infection from infected parents through semen, during pregnancy and by consumption of colostrums and milk (vertical transmission) and oral-faecal route (horizontal transmission) from contaminated environment (soil, water, fodder, feed and pasture).

Pradhan *et al*. (2011) found that the phenomenon of ‘‘passive shedding’’ of MAP in faeces, wherein an animal ingests MAP and subsequently sheds the bacterium in its faeces without becoming infected, is possible, but is more likely in heavily contaminated environments.

Fritsch*et al.* (2012) proposes that interaction of *Mycobacterium avium subspecies paratuberculosis* with susceptible farmed ruminants raises the possibility of playing a role in the epidemiology of the disease in domestic livestock. An interspecies transmission between cattle and wild-living red deer (*Cervus elaphus*) has been recently proposed by multitarget genotyping of strains from both animal species.

2.4 Diagnosis

Yokomizo *et al*. (1983) stated the immunological test that is widely available and commonly used is the Enzyme Linked Immunosorbant Assay (ELISA), which detects antibody in serum that correlates with an antibody response to MAP. Paratuberculosis is essentially untreatable (Chiodini *et al*., 1984)

Collins *et al.* (1989) found that insertion sequence IS900, discovered in the late 1980s, is most widely used genetic marker for specific detection of MAP. DNA probes based on IS900 enable detection of MAP without cultivating bacteria, hence are faster (in less than three days). DNA probes are costly and difficult to perform.

Vary *et al.* (1990) found the PCR technique offers specific and rapid detection of *Mycobacterium avium* subspecies *paratuberculosis*, However,, reduced levels of sensitivity have been encountered when it has been applied on clinical samples, and these reductions have been attributed to the presence of inhibitors.

Collins *et al.* (1989) reported usual mix of animals (sub-clinical, clinical and advance clinical) in a herd renders herd level sensitivity of ELISA about 45% and faecal culture about 45-55% (Socket *et al*., 1992).

Socket *et al.* (1992) observed although the specificity of the faecal culture method is high, the organism can take 12 to 16 weeks to grow to detectable levels, and even the most sensitive culture methods have less than 50% sensitivity .

Collins *et al.* (1996) reported that Serologic tests such as agar gel immunodiffusion, complement fixation , and enzyme-linked immunosorbent assay (ELISA) are limited in their use because of both low specificity and sensitivity.

Collins *et al*. (1996) observed that serological tests are of limited value due to low specificity and sensitivity, since antibodies may not be detectable either due to anergy or until their late appearance in the pathogenesis of Johne's disease .

Mishina *et al.* (1996) stated that IS900 PCR has frequently been used to confirm MAP in cases of Crohn's disease and controls.

Sweeney *et al*.(1996) found that the only effective control measures are farm management strategies aimed at the reduction of MAP transmission and the culling of infected deer, which requires an accurate diagnostic test for detection .

Onet (1997) studied bacteriological culture of MAP, with its relatively low sensitivity but high specificity, has been considered the definitive diagnostic test for MAP infection. Culture of tissue in particular is regarded as the post mortem diagnostic "gold standard.

Englund *et al*. (1999) reported that higher sensitivity observed could be due to the use of tissue samples instead of faecal extracts, which are known to contain PCR inhibitors.

Cousins *et al.* (1999) stated PCR is less sensitive than culture when applied to screen clinical samples due to presence of inhibitory substances or non-recovery of DNA. Another concern is non-specificity of IS900 due to presence of IS900 like sequences in non-MAP mycobacteria.

Restriction endonuclease analysis (REA) of the insertion sequence IS1311 was carried out on all MAP strains isolated, to discriminate sheep and cattle strains, according to the technique described in Marsh *et al*. (1999).

Schwartz *et al.* (2000) identified the role of MAP in causation of CD has been supported by the frequent isolations of MAP from the CD patients as compared to

other suspected and ulcerative colitis (UC) patients, detection of MAP RNA in biopsies of CD patients, immunological response and response to anti mycobacterial therapy by CD patients. However,, critics of the above theory claimed MAP to be environmental or normal intestinal commensal and CD is the result of molecular mimicry between intestine.

Corti *et al.* (2002) suggested that Nucleic acid amplification [Polymerase chain reaction (PCR)] provides a rapid alternative for the specific diagnosis of MAP infection. IS*900* element (present in 14-18 copies in MAP genome)-based PCR protocols have been optimized and most frequently used for the specific detection of MAP in clinical samples (blood, faeces and milk).

Rideout *et al*. (2003) reported that culture being 100 percent specific is considered 'Gold standard' for the diagnosis of MAP infection in animals and requires 12-16 week of incubation .

National Academy of Sciences Report **(**2003) states isolation of *Mycobacterium avium* subspecies *paratuberculosis* in conjunction with histopathological lesions is regarded as the gold standard for diagnosis of Johne's disease.

Robino *et al*. (2003) stated that Deoxyribonucleic acid (DNA) was extracted from colonies and lymph nodes following a modified protocol for mycobacteria and MAP was identified using IS900 nested polymerase chain reaction (PCR).

According to Glanemann *et al.* (2004) and Collins *et al.* (2005), immunological-based systems are faster than culture methods but have a low sensitivity.

Shin *et al.* (2007) stated that use of new techniques (BACTEC system, MGIT system and MB Bactec system) help in the rapid detection of MAP in culture;

However, high cost of equipment and biochemicals limits the use of these techniques, especially in resource-poor countries including India (Gumber *et al*.,2007).

Singh *et al.* (2007) reported that IS900 PCR is the most widely used molecular test to diagnose MAP infection. When applied to confirm MAP colonies IS900 PCR has 100% specificity and sensitivity. PCR assays have also been optimized to screen clinical samples (tissues, blood, milk and faeces). Sohal *et al.* (2007) reported high sensitivity for serological tests and faecal culture is only possible when animals are in clinical stage of disease.

Khare *et al*. (2008) reported that sample storage conditions can affect MAP viability and the subsequent ability to culture.Coelho *et al*. (2008) suggest that smears stained with Ziehl-Neelsen are good indicators of infection and could be used to easily and rapidly screen tissue for the presence of compatible organisms that could be confirmed later by other methods with the added advantage of being inexpensive and fast.

Castellano *et al.* (2012) investigated diagnosis based on the detection of IS*900*-specificsequences of MAP by polymerase chain reaction (PCR) from tissue, faeces and blood is considered to be very quick and highly specific.

Preziuso*et al.* (2012) reportedthat diagnosis based on the detection of IS*900*-specificsequences of MAP by polymerase chain reaction (PCR) from tissue, faeces and blood is considered to be very quick and highly specific and the most sensitive technique for detecting MAP, especially in extra-intestinal samples.

Wadhwa *et al.* (2012) reported a wide range of serological tests such as enzyme-linked immune sorbent assay (ELISA), agar gel immunodiffusion, delayed type hypersensitivity, interferon-gamma assay, fluorescence antibody test and complement fixation test successfully used for the detection of MAP infection. Of the four tests, microscopy and ELISA have been found to be good screening tests, and

culture and PCR as confirmatory tests (Sonawane *et al*., 2013). For chronic and insidious disease such as MAP infection, it is recommended to use multiple tests as per the purpose and resources of the animal owner.

Sonawane *et al.* (2013) concluded that diagnosis of MAP infection is difficult, and no single test can diagnose all cases with absolute accuracy.

Garg *et al.* (2015) suggested that available diagnostic tests may either detect the bacilli or the host's immune response. Microscopy of the samples (faeces, milk, tissues and blood) is the most convenient test, which can be performed within limited resources, but heavily depends on the expertise and training of the worker.

2.5 Prevention and Control

Ursing *et al.* (1982) reported that since this disease is transmitted vertically through semen, colostrums and milk to next generation, and becomes endemic in herds and flocks and continues to perpetuate. In case of human infection, the drugs used in the treatment of tuberculosis showed only 50 percent efficacy on MAP.

Welles *et al.* (2000) Control of MAP by treatment of sick animals is neither practical nor cost-effective since treatment cost may run over the cost of animal. Traditional method of control and management of disease (JD) in animals, based on 'test and cull' policy, besides being expensive failed to control disease.

Allworth *et al*. (2000) and Groenendaal *et al*. (2003) stated that due to concerns about animal health, economic considerations, and zoonotic potential of paratuberculosis, several countries and many states within the United States have instituted Johne's disease certification programs for prevention and control of the disease.

Stabel *et al*. (2004) reported prevention and control of Johne's disease is severely hindered due to its long incubation period, presence of undetected subclinical cases, absence of rapid *Mycobacterium avium* subspecies *paratuberculosis* specific diagnostic tools and efficacious vaccines, and lack of knowledge of strain diversity.

Collins (2010) stated that there is no treatment for this disease in any species.

CHAPTER-III

MATERIALS AND METHODS

The present study on "**Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection among ruminants in Nandanakanan Zoological Park(NZP), Odisha"** was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry ,Orissa University of Agriculture and Technology, Bhubaneswar from January 2018 to June 2018 using laboratory facilities available in Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, Farah,Mathura, UP , India.

3.1 Sources of animals and bio-samples

Wild small ruminant of Nandandankanan Zoological Park were selected for the study. All the wild ruminants were under chemo-prophylactic measure against tuberculosis i.e. Mycocox-E for 3 months in a year. A total of 17 faecal samples from 10 species i.e Mouse deer (2 samples), Manipuri deer (2 samples), Barking deer (2 samples), Chousinga (1 samples), Black buck (1 samples), Spotted deer (5 samples), Hog deer (2 samples), Nilgai (1 samples), Barasinga (1 samples), White buck (1 samples) were collected. 25 each blood and serum samples were collected from apaparently healthy spotted deer. Samples were screened for detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection by Ziehl-Neelsen (ZN staining) , indirect ELISA and molecular identification of MAP by PCR.

3.2 Collection of bio-samples

3.2.1 Collection of faecal samples

Faecal samples were collected either directly from rectum or when freshly passed stool of animals in a sterilized container. About 2 gm (2-4 pellets), of the faeces were taken and brought to laboratory for identification of MAP.

3.2.2 Collecction of blood samples

Blood samples were collected from jugular vein of tranquilised deer (Tranquilisation is done by air gun- Dan Inject with xylazine-ketamine cocktail from a distance about 40 meter) in 5 ml Dispovan with 20 gauge needle Half of these blood were transferred to container with anticoagulant and another half is transferred to container with clot activator for harvesting of serum.These samples container were immediately brought to laboratory at 4^0C for identification of MAP .

3.3 Diagnostic tests used for MAP detection

The test employed for detection of MAP infection were as follows: ZN staining (Singh *et al*.,2013), indirect ELISA(Singh *et al*.,2007) and PCR (Singh *et al*.,2010).

3.3.1 Ziehl–Neelsen staining

This test was performed following the procedure described by Singh *et al*.(2013).About 2 gram of faecal sample was finely crushed in the sterilized 'pestle and mortar',with 10-15 ml sterilized distilled water and turned into fine paste. This sample material was transferred to 15 ml centrifuge tubes and centrifuged at 4500 rpm for 45 min at room temperature to concentrate the bacilli. Supernatant was discarded and smear were prepared on a grease free glass slide from middle layer.Then it is air dired and heat fixed for staining.

For ZN staining, stain was purchased from HIMEDIA.The slides were heat fixed and were flooded with carbol-fushin and heated gently till steaming and were left for 10-12 minutes. Slides were washed in tap water and de-stained with acid alcohol for

30 seconds and the step was repeated till the pink colour appeared coming. Slides were counter stained with methylene blue for 30 sec and final washing is done with tap water. Ziehl–Neelsen-stained smears were air dried and examined for the presence of acid fast bacilli under oil immersion objective. Pink coloured short rods (0.5–1.5 µm) were considered as positive for MAP infection. Nearly 10 fields were seen of each samples were seen and ratings were given as per Table.1

Table 1. Interpretation of ZN staining

Sl.No.	Observation(in 10 fields)	Ratings	Interpretation
1	10 bacilli or one bunch in one field	+1	Low shedder
2	10 bacilli or one bunch in alternate of 2,3 or 4 fields	+2	Moderate shedder
3	10 bacilli or one bunch in each alternate field	+3	Moderate shedder
4	10 bacilli or one bunch in each field	+4	Super shedder

Fig.1. Ziehl–Neelsen staining of faecal sample smear

3.3.2 Indirect ELISA

The test was conducted as described by Singh *et al. (*2007) for indirect ELISA with indirect ELISA kit.

3.3.2.1 Native antigens

For the comparative analysis native semi-purified Protoplasmic antigens (Ns_PPA) were prepared from the novel biotype ('Indian Bison Type') of MAP strain 'S 5' recovered from the advance case of JD in a goat (Sevilla*et al.,* 2005). This strain was isolated from a terminal case of Johne's disease (extremely weak and recumbent) in a Jamunapari goat located at Central Institute for Research on Goats (CIRG), Makhdoom, which later succumbed to JD.

3.3.2.2 Indigenous ELISA kit

Native MAP strain (S 5) characterized as 'Indian Bison Type' of goat origin (Jamunapari goat died due to JD) was used as antigen source (Singh *et al*, 2007, Sohal *et al*, 2007). Soluble protoplasmic antigen prepared from Indian Bison Type genotype of MAP isolated from terminal case of JD in a goat, was used for the screening against MAP by indigenous ELISA (Singh *et al*, 2007).

- Antigen (Indian Bison Type) was coated at 0.1 microgram (in 100 micro litre of carbonate buffer pH 9.6) per well of the microtiter plate.
- It was kept overnight at 4^0C. Then it is washed with PBST thrice. Wells of the plate was blocked with 200 µl of skimmed milk (in PBS) and incubated at 37^0C for one hour.
- It was again washed thrice with PBST.
- 100 µl of serum samples were added in 1:50 dilution to the well (diluted using buffer containing PBST with 1% BSA and 2 mg/ml *M. phelei*) and incubated for 2 hours at 37^0C.

- Plate was washed thrice with PBST.
- Then 100 µl of optimally diluted (1:8000) conjugate, anti-Goat horseradish peroxidase conjugate (Sigma Aldirch, USA) in PBS and incubated for one hour at 37^0C.
- Plate was washed again thrice with PBST.
- Finally 200 µl of freshly prepared substrate (o-phenylene diamine dihydrocholride) at concentration of 5mg per plate in substrate buffer (pH 5.0) to wells of plate.
- Then it was incubated (in dark) for 20 minutes at room temperature.
- OD was taken at 450 nm in ELISA reader (Bio-Rad company) without adding stop solution (5N H_2SO_4).
- Serum samples from culture positive and negative samples of cattle were used as positive and negative controls, respectively. Strong positive and positive samples were considered as positive for MAP infection.

Sample-to-Positive ratio: Optical densities (OD) were expressed as ratios as per Collins (2002) by following calculations.

$$\text{S/P ratio value} = \frac{\text{OD at 450nm of test serum} - \text{OD at 450nm of negative control}}{\text{OD at 450nm of positive control} - \text{OD at 450nm of negative contro}}$$

Table 2. Sample to Positive (S / P) ratios and status of JD on the basis of likelihood ratios

S.No.	S/P Ratios	JD status
1	0.00 – 0.09	Negative
2	0.10 – 0.24	Suspected or Borderline
3	0.25 – 0.39	Low Positive
4	0.4 – 0.99	Positive
5	1.0 – 10.0	Strong Positive

Fig. 2. ELISA optical density Reader

3.3.3 Molecular Identification of MAP(PCR)

Molecular identification of MAP was performed as per description by Singh *et al*. (2010).

Equipments used

- Master-Cycler (Techne 4000)
- Micro-centrifuge (Hermle)
- Digital pH meter (Eutech)
- Analytic Balance (Afcoset)
- Micropipettes (Eppendrof, P'fact)
- Vertex mixture (Labnet)
- Mcrowave oven (Samsung)
- Deep freezer (Blue star)
- Water purification system (ASGI,Agra)
- Gel Electrophoresis system (Gene Mate)
- E-gel imager system (UV transilluminator- Alpha Innotech)
- Chemical and biological agent in PCR from Thermofischer Scientific, Genetix and Biorad company

3.3.3.1 Isolation of DNA from blood

- One milliliter of erythrocyte lysis buffer (320mM Dextrose, 5mM $MgCl_2$, 1% triton X-100, TrisHCl 10mM; pH-7.5) was added to 500 μl blood samples.
- Tubes were vortexed vigorously and spun at 15000 g for 2 min. Pellet containing WBCs was again treated with erythrocyte lysis buffer until the pellet became white.
- The 400 μl of nucleic lysis buffer (60mM NH_4Cl; 24mM Na_2EDTA; 1 mg/mL Proteinase K; pH-8) and 100 μl of 1% SDS were mixed and used to suspend the WBC pellet and incubated at 55◦C in a water bath for 30 minutes.
- After digestion samples were cooled at room temperature and centrifuged at 15000 g for 10 minutes.
- Supernatant was collected and 100 μl of ammonium acetate (3 M) was added and again centrifuged at 15000 rpm for 10 minutes. Supernatant containing genomic DNA of goats and MAP DNA (if present) was transferred to fresh Eppendorf tube.
- A double volume of absolute ethanol was added and tubes were gently inverted several times until DNA was precipitated as threads.
- Tubes were centrifuged at 15000 g for 10 minutes. DNA pellet was washed with 1mL of 70% ethanol, air dried, resuspended in 30 μl of TE buffer (pH 8), and kept at -20^0C for further use. Isolated DNA contained both goat genomic DNA and DNA of MAP.

3.3.3.3Isolation of DNA from faeces

- Semi solid middle layer of the concentrated faecal samples was taken in 400 microliter of sterilized Phosphate buffer saline (PBS) and was subjected to heating at 95°C for 15-20 min and after that 50 µl of Lysozyme (20mg/ml) was added followed by incubation at 37°C for 2 hours in a water bath.
- Then 20 µl of 10% sodium dodecyl sulphate (SDS) and 20 µl of Proteinase K (10 mg/ml) were added, followed by incubation at 56°C for 2 hours in a water bath.

- Then 64 µl of CTAB/NaCl (preheated to 65°C) added and incubated at 65°C for 30 min. After incubation, equal volume of Cloroform-isoamyl alcohol (24:1) was added and vortexed properly.
- The suspension was incubated at room temperature for 5 minutes and was then centrifuged at 10000 rpm for 10-15 minutes.
- After centrifugation aqueous layer containing nucleic acids was transferred to sterilized eppendorf tube.
- After centrifugation aqueous layer containing nucleic acid was transferred to sterilized eppendorf tube.
- To the aqueous layer, 0.6 volumes of isopropyl alcohol and 1/10th of 3M sodium acetate was added and kept at -20°C for overnight to allow precipitation of DNA.
- After precipitation, centrifugation was done at 10000 rpm for 15 min. Supernatant was discarded and sediment was washed twice with 1ml of 70 percent ethanol by centrifugation at 10000 rpm for 10 min. Pellet was allowed to air dry.
- DNA pellet was re-suspended in 30µl TE buffer, then kept at 4°C for 1 hour to allow for dissolution of the pellet and then stored at -20°C for further use (Van Embden *et al* 1993).

3.3.3.3 Checking the purity of DNA

Accurate dilution of DNA (e.g. 1:1000) was made in distilled water and mixed well. Reference cell was filled with 3/4 volume of TE buffer. It was made sure not to leave bubbles that was deflect the light beam and introduce errors. Similarly the other cell was filled with diluted DNA. Cross contamination of samples and reference during pippeting was avoided. Measure the OD at 260 nm and 280 nm by spectophotometer.

$$\text{Purity} = \frac{\text{OD at 260 nm}}{\text{OD at 280 nm}}$$

Ratio between the readings at 260 nm and 280 nm provided estimation of purity of the nucleic acids. Pure preparations of DNA had OD values of 1.8 to 2.0 respectively. If there is contamination with phenol or protein, the OD 260 / OD 280 was be significantly less than the above value. The accurate quantitation of amount of nucleic

acid was not possible. After checking the value of purity, calculated the DNA concentration of given sample by following method:

DNA concentration (μg/ml) = OD_{260} x 100 (dilution factor) x 50 μg/ml /1000

3.3.3.4 Molecular characterization by IS*900* PCR

DNA were screened by IS*900* PCR using P90 and P91 primers as per Singh *et al*. (2010). Briefly, in a volume of 10 μl of 2X PCR master mix (Thermo scientific), 0.5μl forward primer (10 pmole/μl) and 0.5μl reverse primer (10 pmole/μl), 3.5μl of nuclease free water and 3 μl of sample DNA was added (total volume 20 μl).Thermal cycling conditions were: initial denaturation at 94^0C for 3 min, followed by 37 cycles of denaturation at 94^0C for 30 s, annealing at 64^0C for 30s, extension at 72^0C for 1 min, and final extension at 72^0C for 7 min. Amplicon sizes of 413 bp were considered positive, after separation on 2% agarose gel stained with ethidium bromide.

Table 2.Primers used for characterization of *Mycobacterium avium* subsp. *paratuberculosis*

Target	Primer	Primer sequence	Product Size
IS *900*(Millar *et al*. 1996)	Forward: P90	GAA GGG TGT TCG GGG CCG TCG CTT AGG	413 bp
	Reverse: P91	GGC GTT GAG GTC GAT CGC CCA CGT GAC	

Table 3. Reaction mixture for conventional PCR amplication

Sl. No.	Stock Solution	Quantity per samle(µl)
1	10 x Taq Buffer	2.50
2	25mM $MgCl_2$	1.50
3	Taq polymerase(5U/ µl)	0.20
4	2mM dNTP mix	2.50
5	Forward primer	0.5
6	Reverse primer	0.5
7	Template DNA	0.5ng
8	HPLC Water	Variable
9	**Total Volume**	25.0 µl

Table 4. Conditions in PCR cycle

Sl. No.	Steps	Temperature	Time
1	Initial Denaturation	95^{o}C	3 minute
2	Denaturation	95^{o}C	30 second
3	Anneaaling	50^{o}C	30 second
4	Extension	72^{o}C	1 minute
5	Final extension	72^{o}C	7 minute
6	HOLD	4^{o}C forever	

The PCR cycling conditions i.e. denaturation, annealing, extension were carried out for 40 cycles followed by final extension.

3.3.3.5 Agarose gel electrophoresis

Horizontal electrophoresis apparatus consists of buffer chamber and comb, waterbath, microoven, UV transparent gel tray, gel casting platform, gel sealing tape, spirit level and common lab tape. Agarose gel 2% was prepared by taking 40 ml agarose gel to 15 ml of 1X TBE buffer in a conical flask and was melted until it was dissolved

completely. When checked against light the undissolved agarose appeared as small flakes floating in the solution and then ethylene bromide 3µ (10 mg/ml) was added. Casting of gel is done by pouring melted agarose in the tray so that air bubbles are avoided in between the teeth of comb. Then electrophoresis buffers were poured in the gel tray.

Once solidified, the agarose gel is placed into the gel box (electrophoresis unit). Gel box is filled with 1x TBE until the gel is covered. Then 5 µl of PCR product was added to 3 µl of loading dye added. And slowly loaded into each well of the submerged gel.1 KB ladder is added into the first lane of the gel. Then gel is run at 100V for 15 minute. After that the electrodes are disconnected from the power source, and then carefully the gel is removed from the gel box. And it is visualised by UV-light transillumination.

Fig.3. Isolation of DNA from blood sample

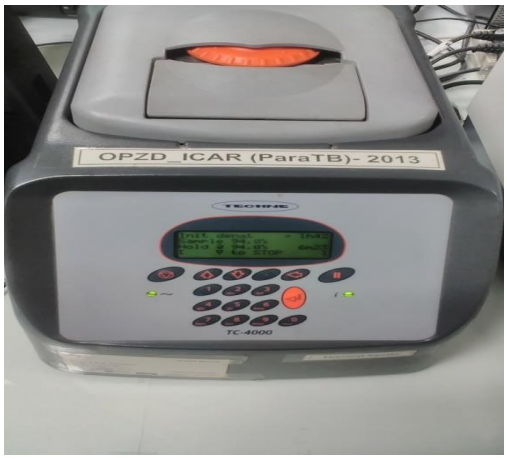

Fig. 4. Amplication of DNA in master cycler

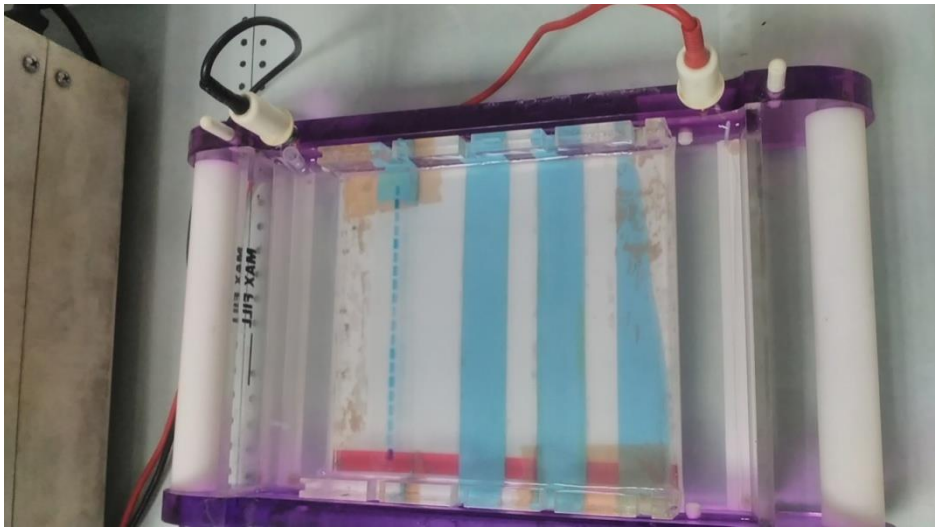

Fig.5. PCR sample products undergoing gel electrophoresis

CHAPTER-IV

RESULTS

The present study on **"Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection among ruminants in Nandanakanan Zoological Park(NZP), Odisha"** was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar from January 2018 to June 2018using laboratory facilities available in Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, Farah, Mathura, UP , India.

4.1 Detection of MAP from ZN staining of faecal smear

A total number of 17 faecal samples collected from 10 wild small ruminant(deer) species which are apparently healthy animal from Nandankanan Zoological Park(NZP), Odisha were subjected to ZN staining. Three out of 17 sample were found positive with +1 rating. The incidence of MAP infection was 17.65%(3/17)(Table.5).

Table 5. Detection of MAP through ZN staining

Sl. No.	Wild Ruminant Species	Scientific Name	Result for ZN staining
1	Spotted deer-1	*Axis axis*	Negative
2	Spotted deer-2	*Axis axis*	Negative
3	Spotted deer-3	*Axis axis*	Negative
4	Spotted deer-4	*Axis axis*	Negative
5	Barking deer-1	*Muntiacus muntjack*	Negative
6	Barking deer_2	*Muntiacus muntjack*	Negative
7	Manipuri deer-1	*Rucervus eldii eldii*	Negative
8	Manipuri deer-2	*Rucervus eldii eldii*	Negative
9	Mouse deer-1	*Moschiola indica*	Negative
10	Mouse deer-2	*Moschiola indica*	Negative
11	Hog deer-1	*Axis porcinus*	Positive(+1)

12	Hog deer-2	*Axis porcinus*	Negative
13	Barasingha	*Rucervus duvaucelli*	Negative
14	Black buck	*Antilope cervicapra*	Negative
15	Nilgai	*Boselaphus tragocamelus*	Negative
16	White buck	*Antilopecervicapra*	Positive(+1)
17	Chousinga	*Tetracerus quadricornis*	Positive(+1)
	Total positive		**3**

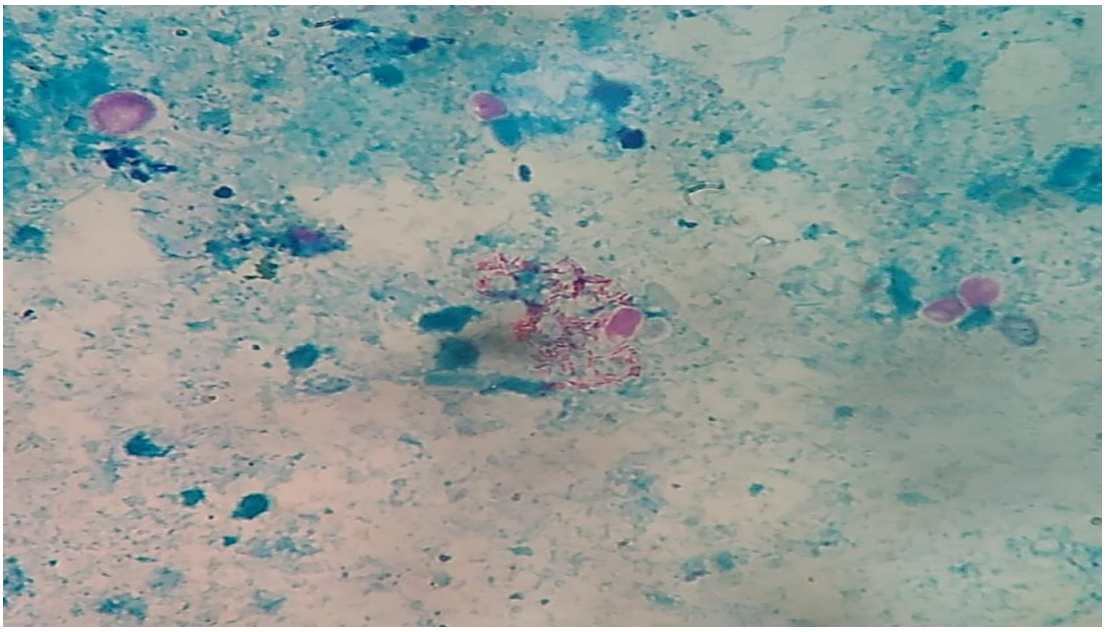

Fig. 5. MAP bacilli detected through ZN staining in Chousinga

4.2 Detection MAP antibody by indigenous i_ELISA

Twenty five serum samples of spotted deer collected from Nandankanan Zoological Park were subjected to i_ELISA. Four out of 25 samples were found as positive, 5 samples were low positive, 13 samples were suspected and 2 samples were negative(Table.6). Incidence of MAP was 19.2%. However, no animals were found strong positive. Samples were also collected from apparently healthy animals. Cases, whether positive, negative or suspected were decided on the basis of S/P ratio.

Table.6 Detection of MAP antibody through i_ELISA

Sl.no.	Species	S/P ratio	Remark
1	Spotted deer-1	0.212	Supected
2	Spotted deer-2	0.085	Negative
3	Spotted deer-3	0.228	Supected
4	Spotted deer-4	0.195	Supected`
5	Spotted deer-5	0.463	Positive
6	Spotted deer-6	0.315	Low positive
7	Spotted deer-7	0.479	Positive
8	Spotted deer-8	0.454	Positive
9	Spotted deer-9	0.174	Supected
10	Spotted deer-10	0.194	Supected
11	Spotted deer-11	0.184	Supected
12	Spotted deer-12	0.056	Negative
13	Spotted deer-13	0.112	Supected
14	Spotted deer-14	0.169	Supected
15	Spotted deer-15	0.228	Supected
16	Spotted deer-16	0.304	Low positive
17	Spotted deer-17	0.454	Positive
18	Spotted deer-18	0.328	Low positive
19	Spotted deer-19	0.197	Supected
20	Spotted deer-20	0.290	Low positive
21	Spotted deer-21	0.149	Supected
22	Spotted deer-22	0.275	Low positive
23	Spotted deer-23	0.237	Supected
24	Spotted deer-24	0.192	Supected
25	Spotted deer-25	0.203	Supected
	Total positive		**4**

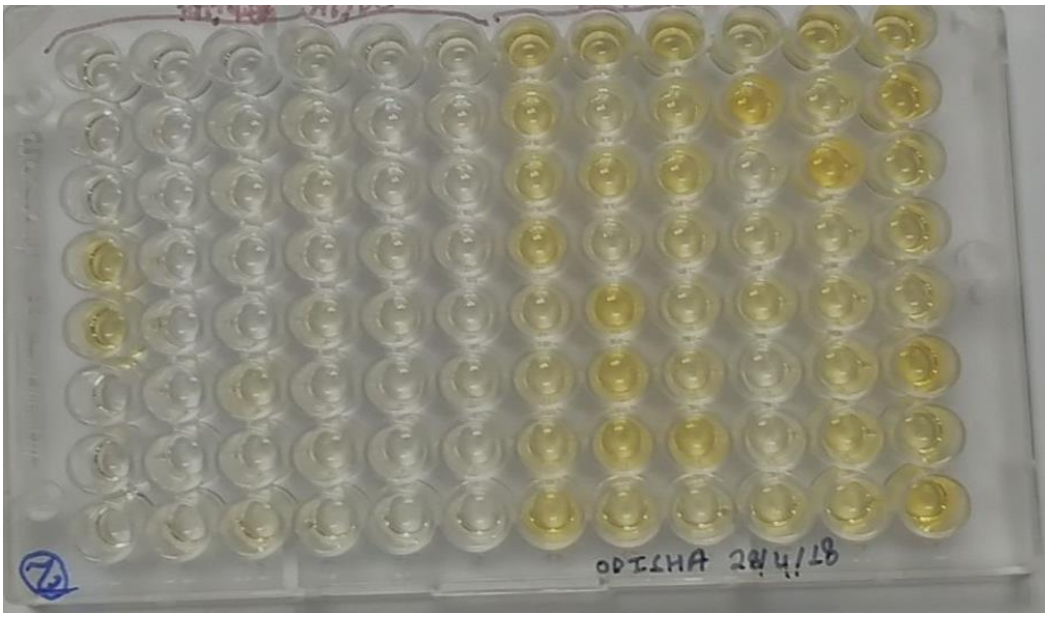

Fig. 6. ELISA reaction

4.3 Detection of blood antigen and faecal antigen by PCR reaction

A total number of 25 anticoagulated blood samples were collected from ruminants of Nandankanan Zoological Park, Odisha. 4 samples showed positive reaction for ELISA were again subjected to PCR. Along with 13 negative samples for ELISA were also subjected to PCR. But none of them were found positive after gel electrophoresis of PCR product (Table.7). Three out of 17 faecal samples were positive for MAP. These 3 faecal samples were also subjected to PCR. These sample were found positive after gel electrophoresis of PCR product(Table.7).

Table.7 Sample result for PCR reaction

Sl.no.	Species	Type of Antigen DNA	Remark
1	Mouse Deer-2	Faecal antigen DNA	Negative
2	Hog Deer-1	Faecal antigen DNA	Negative
3	White buck	Faecal antigen DNA	Negative
4	Chousinga	Faecal antigen DNA	Negative
5	Spotted deer-5	Blood antigen DNA	Negative
6	Spotted deer-6	Blood antigen DNA	Negative
7	Spotted deer-7	Blood antigen DNA	Negative

8	Spotted deer-8	Blood antigen DNA	Negative
9	Spotted deer-9	Blood antigen DNA	Negative
10	Spotted deer-10	Blood antigen DNA	Negative
11	Spotted deer-11	Blood antigen DNA	Negative
12	Spotted deer-14	Blood antigen DNA	Negative
13	Spotted deer-16	Blood antigen DNA	Negative
14	Spotted deer-17	Blood antigen DNA	Negative
15	Spotted deer-19	Blood antigen DNA	Negative
16	Spotted deer-20	Blood antigen DNA	Negative
17	Spotted deer-21	Blood antigen DNA	Negative

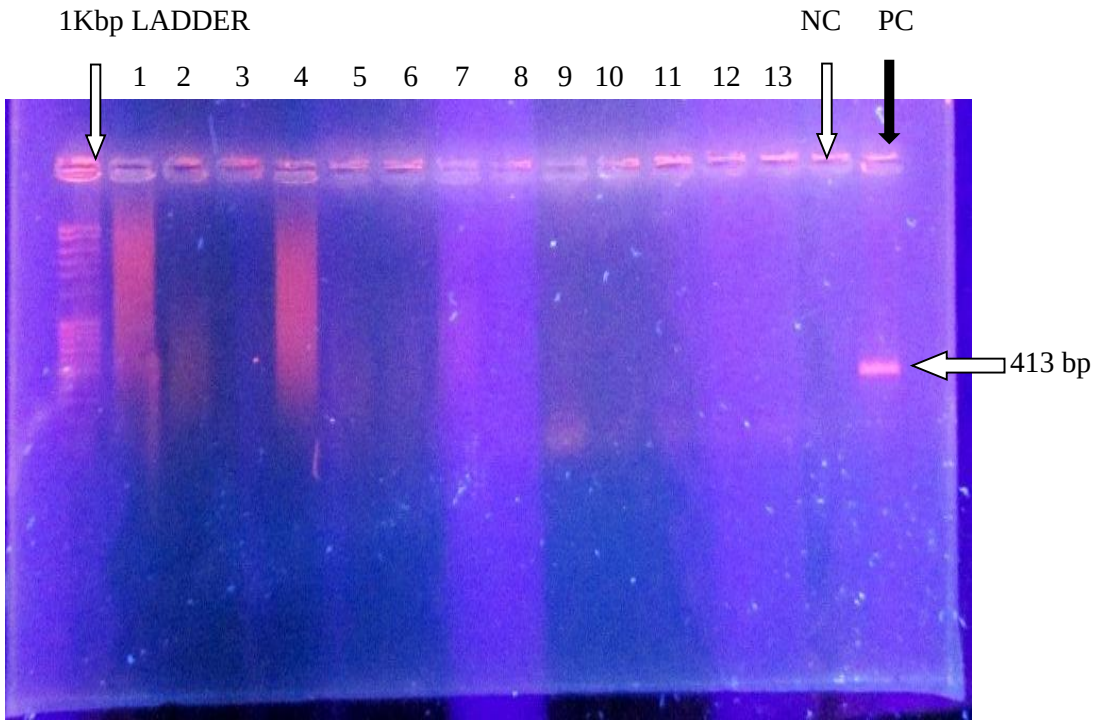

Fig.8.Agarose gel electrophoresis of PCR product

CHAPTER-V

DISCUSSION

Mycobacterium avium subspecies *Paratuberculosis* infection is an economically disruptive herd health problem for domestic ruminant species. The infection results in Johne's disease, a chronic granulomatous gastroenteritis leading to emaciation and death. Cases of the infection have been also reported in a number of free-ranging ruminant species (Williams *et al*.,2001). Paratuberculosis has been reported to occur in wild ruminant species (deer, bison, and elk). Paratuberculosis was also reported in nonruminants, wild rabbits, their predators (including foxes and stoats) and primates (mandrills and macaques) indicating a wide host range. The disease infections in free-ranging and captive wildlife is also of great concern. Up to one-third of zoo accredited by the American Zoo and Aquarium Association have reported at least one culture-confirmed case of paratuberculosis since 1995.In addition, *Mycobacterium paratuberculosis* is of great interest because of its potential association with Crohn's disease in humans. The existence and importance of wildlife reservoirs of *M. paratuberculosis* in the transmission cycle is still undetermined and a few investigators have examined the role of wildlife in the epidemiology of this important disease (Motiwala *et al*.,2004).

In this work three types of diagnostic methods were followed: ZN staining, i_ELISA kit and IS*900*PCR.

From 17 samples, 17.6%(3?17) were found positive. These findings are somewhat similar to Singh *et al*.(2010) who reported 20 %(4/20) prevalence of MAP in Hog deer (*Axis porcinus*). Erume *et al*. (2001) detected higher percentage 48.5% of MAP by ZN staining of faecal sample of captive wild animals of Zoo.

Twenty five serum sample were subjected to i_ELISA from which 15.3%(4/26) were found positive for MAP. However, these findings are somewhat similar to findings by Murray *et al*.(2008) who found 12.5%(2/16) from 16 farmed white tailed deer.

For IS900 also different primer IS900/P36 (59-GGCCGTCGCTTAGGCTTCGA-39) and IS900/P11(59-CGTCGTTAATAACCATGCAG-39) were suggested by Moss *et al*. (1991). However, Marsh primer was used in this study. 4 faecal samples DNA and 13 blood samples DNA were subjected to PCR. But none of them were found positive. These findings are akin to the findings by Erume *et al*. (2001) who observed 2 samples were negative which was found positive for MAP in nested PCR.

Individual deer were classified as infected with MAP if any one of the culture slants from their faeces, lymph nodes, or ileum were positive, or if a CS-PCR or direct PCR assay detected MAP DNA in any of their tissues. Deer, identified as positive for MAP by indirect tests such as AGID and ELISA, but not by bacterial culture, Ziehl-Neelsen staining, or PCR assay were classified as suspected cases (Murray *et al*.,2008).

Because of only 3 samples were found positive as low shedder in ZN staining,it is evident that animals are not clinically affected. It indicates the shedding of MAP organism in faeces before appearance of clinical sign (Lepper *et al*.,1989). In heavily contaminated environments chances of passive shedding may be there. In this phenomenon animals ingest the bacterium and ingested bacterium is shed in the faeces without infecting the animal (Pradhan *et al*.,2011). Therefore, in this case passive shedding may not be there because of detection of low prevalence of MAP.

In this study, 4 samples were positive by serological test ELISA. They were not strong positive. This ELISA-positive cases can only be confirmed by culture of mycobacterium from faecal sample.

All the PCR reactions were found negative. Detection of MAP by IS*900* PCR in faecal samples was rapid, but has low through-put as MAP shedding at early and sub-clinical stages is low and intermittent (Shah *et al*.,2012). From the ZN staining we concluded that animals were low shedder. So reason for all negative PCR reaction agreeing to the above statement by Shah *et al*. (2012).

Less number of sample were used in this study. But more sample is needed for clear interpretation. Collection of ante-mortem sample in captive wild ruminant like deer is difficult because of capture myopathy.

Culture of MAP being 100 percent specific is considered 'Gold standard' for the diagnosis of MAP infection in animals and requires 12-16 week of incubaction (Rideout *et al*.,2003). We had also performed inoculation of sample in Herold Egg Yolk medium with mycobactin for primary isolation of MAP as follows:

There are 3 types of MAP strain

1)Type –I (Sheep type) found in sheep

2)Type-II (Cattle type) and B type(Bison Type) found in sheep, goat, ruminant, nonruminant, human and wild animal.

3)Type-III (Intermediate type)

MAP unable to synthesize mycobactin depends on it in primary isolation. Type-II strains are found in wild animals. So for primary isolation Herold's Egg yolk medium with mycobactin was used. Initially 2gm faeces were crushed in pestle and mortar and solution is made with 10-15ml distilled water. Then it was transferred to 15 ml centrifuge tube and centrifuged at 4500 rpm for 45 minutes. Then top layer was discarded. Middle semisolid layer was collected by sterilised swab and subjected to decontamination at the top of 40 ml of 0.9% Hexadecyl pyridinium chloride for 12-24 hour at room temperature. Herold egg yolk medium was prepared and supplemented with mycobactin (HEYM) and dispensed in MacCartney bottles. These bottles were kept at slanting position.0.2ml of sediment from decontamination tube was taken and inoculated on slants of HEYM. It was kept for 4-5 days at 37^0C with loose cap in slanted

position. After that caps were made tight and lifted to vertical position and incubated for 120 days. Observation of the slant were made at 15 days interval.

Though the inoculation was performed but results of the tests could not be corroborated because of paucity of time. However, it may be done for confirmation of MAP infection.

MAP has been reported from cases of Crohn's disease worldwide. Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are complex disorders reflected by wide variations in clinical practice. IBD has long been considered to affect the populations of North America and Europe more than those of Asia. Crohn's disease was considered almost non-existent in India until 1986. In the last 10years, however, Crohn's disease has been reported more frequently from different parts of India, especially the south. Abdominal ailments are the leading causes of sickness and death in large sections of the 1.2 billion plus human population. Recently very high prevalence of ulcerative colitis has been reported from Northern India (Sood *et al*.,2003) Pain and frequent bouts of diarrhoea overlaps clinical signs in most of the cases of abdominal illnesses.

Medical personnel need to be warned on prevalence of Johne's disease so that they will be tempted to rule out the disease. We have collected blood sample from spotted deer. In future, blood sample can be collected from captive wild ruminants when opportunity comes and it can be analyzed. This can be correlated with as the animals are treated with above drugs it indicates low shedding of MAP in faeces which may be corroborated with the administration of drug i.e. Mycocox-E

CHAPTER-VI

SUMMARY AND CONCLUSION

The present study on "**Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection among small ruminants in Nandanakanan Zoological Park(NZP), Odisha"** was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry under the Orissa University of Agriculture and Technology from January 2018 to June 2018 using the laboratory facilities available in Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, Farah,Mathura, UP , India. Paratuberculosis is a chronic disease of animal. It causes production loss as well as it causes Crohn's disease in human. Nandankanan Zoological Park being a place where many people pays visit in a single day, chances are there acquiring the MAP infection by public, care taker and other animal in zoo. Hence the attempt was made to access the current status of MAP infection among ruminants in NZP, Odisha.

Seventeen faecal samples were subjected to ZN staining. Three out of 17 samples were found positive with +1 rating. It showed the presence of MAP infection in subclinical stage. The prevalance of MAP infection is 17.6%.

Twenty five serum samples were subjected to indigenous indirect ELISA test by i_ELISA kit developed by CIRG, Makhdoom, UP, India. Four out of 25 sample (15.3%) were found positive. Since ELISA was a screening test, the positive samples detected through i_ ELISA and in ZN staining technique were subjected to PCR. Along with these samples 9 more negative sample detected through i_ELISA are also subjected to PCR.

So total of 17 samples (4 faecal and 13 blood) were subjected to PCR assay. No sample were found positive for MAP infection. It indicate sample we have collected were not from clinically infected animal.

It may concluded from the above study that

- Examination of faecal sample and serum sample of small ruminants revealed 17.6 % prevalence and 15.3% seroprevalence of MAP in small ruminants.
- Negative reaction for PCR revealed its low sensitivity and standardisation of PCR is needed for wild ruminant.
- The study indicate the presence of subclinical infection in the Chousinga, White buck and Hog deer of NZP.

REFERENCES

Allworth MB and Kennedy DJ**.** 2000. Progress in national control and assurance programs for ovine Johne's disease in Australia. *Veterinary Microbiology*,**77:** 415-422.

Ambrose NS, Allan RN, Keighley MR, Burdon DW, Youngs D and Barnes P.1985.Antibiotic therapy for treatment in relapse of intestinal Crohn's disease. *Diseases of Colon and Rectum*,**28**: 81-85.

Ana CM, Luis F, Maria HM, Manuela M, Sofia A, Andreia M, Maria LP and Ana CC.2017.Detection of *Mycobacterium avium* subspecies paratuberculosis in kidney samples of red deer (*Cervus elaphus*) in Portugal: Evaluation of different methods.*The Journal of Veterinary Medical Scence*.**79**(3): 692–698.

Ayele WY, Svastova P, Roubal P, Bartos M andPavlik I. 2005. *Mycobacterium avium* subspecies *paratuberculosis* cultured from locally and commercially pasteurized cow's milk in the Czech Republic. *Applied and Environmental Microbiology*.**71**(3): 1210-1214

Ayele YW, Machackova and Pavlik I.2001. The transmission and impact of paratuberculosis infection in domestic and wild ruminants. Journal of Veterinary Medicine,**46**: 205-24.

Barbaruah and Joseph.2008. India: Dairy Giant Walking Barefoot, FAO-RAP Technical Meeting: 17-20.

Bastida F and Juste RA.2011. Paratuberculosis control: a review with a focus on vaccination. *Journal of Immune Based Therapies and Vaccines*:8-9.

Behr MA and Kapur V.2008.The evidence for *Mycobacterium paratuberculosis* in Crohn's disease. *Current Opinion in Gastroenterology*,**24**: 17-21.

Biswal S, Pany SS, Sahoo N, Singh M and Singh SV.2018. Seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Goat Population of Bhubaneswar, Odisha, India. *International Journal of Current Microbiology and Applied Sciences*, **7**(1): 1618-1623.

Buergelt CD, Williams BS, Monif GRG, Pinedo P, Decker JH.2006. Nested polymerase chain reaction and prenatal detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in bovine allantoic fluid and fetuses. *International Journal of Applied Research in Vterinary Medicine***,4**: 232-238.

Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, Rhodes G, Pickup R and Hermon-Taylor J**.**2003. Detection and verification of *Mycobacterium avium* subspecies *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease.*Journal of Clinical Microbiology*, **41:**2915–2923.

Castellanos E, de Juan L, Domínguez L and Aranaz A.2012. Progress in molecular typing of *Mycobacterium avium* subspecies paratuberculosis. Research in Veterinay Science,**92**: 169–179.

Chan-Mcleod ACA, White RG and Russell DE.1995. Body mass and compositioncharacterization of *Mycobacterium avium subspecies paratuberculosis* using IS 900 PCR. International Journal of Experimental Biology**45**: 812–816.

Chaubey KK, Gupta RD, Gupta S, Singh SV, Bhatia AK and Jayaraman S.2016. Trends and advances in the diagnosis and control of paratuberculosis in domestic livestock. *Veterinary Quarterly*,**10**: 1-26.

Chiodini RJ, van Kruiningen HJ and Merkal RS.1984. Ruminant paratuberculosis (Johne's disease): The current status and future prospects. *Cornell Vet1984*,**74**: 218–262.

Chiodini RJ, Van Kruiningen HJ.1983. Eastern white tailed deer as a reservoir of ruminant paratuberculosis. *Journal of the American Veterinary Medical Associaton*.**182**:168-169.

Cocito C, Gilot P, Coene M, De Kesel M, Poupart P and Vannuffel P. 1994. Paratuberculosis. *Clinical Microbiology Review*.**7**(3): 328-45.

Coelho AC, Pinto ML, Coelho AM, Rodrigues J and Juste R.2008. Estimation of the Prevalence of *Mycobacteriumavium*subspecies p*aratuberculosis*by PCR in Sheep Blood. *Small Ruminant Research*,**76**: 201–206.

Cohavy O, Harth G,Horwitz M, Eggena M, Landers C and Sutton.1999. Identification of a novel mycobacterial histone H1 homologue (HupB) as an antigenic target of pANCA monoclonal antibody and serum immunoglobulin A from patients with Crohn's disease. *Infection and Immunity*,**67** : 6510

Collins DM, Gabric DM and de Lisle GW.1989. Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*.*FEMS Microbiology Letters*, **51** :175.

Collins MT, Kenefick KB, Sockett DC, Lambercht RS, McDonald J and Jorgensen JB.2010.Enhanced radiometric detection of *Mycobacterium paratuberculosis* by using filter-concentrated bovine faecal specimens, *Journal of Clinical Microbiology*,**28**: 2514.

Collins MT**.**1996. Diagnosis of paratuberculosis in R. W. Sweeney (ed.). The veterinary clinics of North America food animal practice. Paratuberculosis (Johne's Disease). W. B. Saunders Co., Philadelphia :357–371.

Collins MT.1997. *Mycobacterium paratuberculosis*: a potential food borne pathogen? *Journal of Dairy Science*, **80**: 3445-3448.

\Corn JL, Manning EJB, Sreevatsan S, Fischer JR.2005. Isolation of *Mycobacterium avium* subspecies *paratuberculosis*from free-ranging birds and mammals on livestock premises. *Applied and Environmental microbiology*,71:6963–6967.

Corti S and Stephan R.2002. A survey of the prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in bulk-tank milk samples all over Switzerland. *Proceedings of the 7th International Colloquium on Paratuberculosis*:106.

Cousens PM.2004.Model for Immune Responses to *Mycobacterium* in Cattle.*Infection and Immunity*,**72**(6), 3089- 3096.

Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ and Kluver P.1999. Mycobacteria distinct from *Mycobacterium avium subspecies paratuberculosis*isolated from the faeces ofruminants possess IS900-like sequences detectable IS900 polymerase chain reaction: implications for diagnosis, *MolecularCell Probes*, **3** : 431

Daniels MJ, Hutchings MR, Beard PM, Henderson D, GreigA,Stevenson K and Sharp JM.2003. Do non-ruminant wildlife pose a risk of paratuberculosis to domestic livestock and vice versa in Scotland? *Journal of Wildlife Diseases*,**39**:10–15.

Dargatz DA, Byrum BA, Barber LK, Sweeney RW, Whitlock RH, Shulaw WP, Jacobson RH, and Stabel JR**.** 2001. Evaluation of acommercial ELISA for diagnosis of paratuberculosis in cattle. *Journal of American Veterinary Medical Association*, **218:**1163-1166.

De Lisle GW, Yates GF, Collins MD. 1993. Paratuberculosis in farmed deer: case reports and DNA characterization of isolates of *Mycobacterium*paratuberculosis. *Journal of Veterinary Diagnostic Investigation*,**5**:567-571.

De Meurichy W, Portaels F, Hoorens J, Bauwens L. 1985. Outbreak of a paratuberculosis-like disease in a captive pudu herd (Pudupudu). *Verhandlungsbericht des international symposium uber die Erkrankungen der Zootierre*: 469-480.

Donaghy JA, Totton NL and Rowe MT.2004. Persistence of *Mycobacterium paratuberculosis* during manufacture and ripening of cheddar cheese. *Applied and Environmental Microbiology*.**70**(8): 4899-4905

Eisenberg SW, Koets AP, Hoeboer J, Bouman M, Heederik D and Nielen M.2010. Presence of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples collected on commercial Dutch dairy farms. *Applied and Environmental Microbiology*,**76**: 6310-6312.

Ellingson JL, Anderson JL, Koziczkowski JJ, Radcliff RP, Sloan SJ, Allen SE and Sullivan NM. 2005a. Detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR. Journal of Food Protection.**68(**5): 966-972

Englund S, Ballagi-Pordány A, Bölske G, Johansson KE .1999. Single PCR and Nested-PCR with a mimic molecule for detection of *Mycobacterium avium* subspecies *paratuberculosis. Diagnostic Microbiology and Infectious Diseases*, **33**:163–171.

Erume J, Spergser J and Rosengarten R.2001.Rapid detection of *Mycobacterium avium* subsp. *Paratuberculosis* from cattle and zoo animals by Nested PCR.*African Health Sciences*,**1**(2): 83-89.

Fawcett AR, Goddard PJ, McKelvey WAC, Buxton D, Reid HW, Greig A, MacDonald AJ. 1995. Johne's disease in herd of farmed red deer. *Veterinary Record*, **136**:165-169.

Fecteau ME, BedeniceD, Cebra CK, Pinn TL, Adams SC, Fyock TL, Whitlock RH and Sweeney RW. 2013. Prevalence of *Mycobacterium avium* subspecies in fecal shedding in Alpacas presented to veterinary hospitals in the United States. *Journal of Veterinary Internal Medicine,***27**:1228-1233.

Fritsch I, Luyven G, Kohler H., Lutz W, Mobius P.2012. Suspicion of *Mycobacterium avium* subspecies *paratuberculosis* transmission between cattle and wild-living red deer (*Cervus elaphus*) by multitarget genotyping. *Applied and Environmental Microbiology*, **78**: 1132-1139.

Garg R, Patil PK, Singh SV, Sharma S, Gandham RK, Singh AV, *et al*. Comparative evaluation of different test combinations for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infecting dairy herds in India. *Biomed Research International*,983-978.

Gilmour N and Nyange J .1989. Paratuberculosis (Johne's disease) in deer. *In Practice*,**11**: 193–196.

Glanemann B, Hoelzle LE., Bogli-Stuber K, Jemmi T, Wittenbrink MM: Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss dairy cattle by culture and serology. *Schweizer Fur Archv Tierheilkunde*, **146**: 409-415.

Gortazar C, Ferroglio E, Hofle U, Frolich K, Vicente J.2007. Diseases shared between wildlife and livestock: a European perspective. *European Journal of Wildlife Research* ,**54**:357-360.

Grant IR, Ball HJ and Rowe MT.2002a. Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy

processing establishments in the United Kingdom. *Applied and Environmental Microbiology*,**68**(5): 2428-2435.

Grant IR,Williams AG, Rowe MT and Muir DD.2005. Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium subspecies paratuberculosis* in milk. *Applied and Environmental Microbiology*,**71**(6):2853-2861.

Greenstein RJ and Collins MT.2004. Emerging pathogens: is *Mycobacterium avium* subspecies *paratuberculos* is zoonotic ?, **364**: 396-397.

Greig A, Stevenson K, Henderson D, Perez V, Hughes V, Pavlik I, Hines II ME, McKendrick I and Sharp JM. 1999. Epidemiological study of paratuberculosis in wild rabbits in Scotland. Journal of Clinical Microbiology, **37**(6): 1746-1751.

Groenendaal H, Nielen M and Hesselink JW.2003. Development of the Dutch Johne's disease control program supported by a simulation model. *Preventive Veterionary Medicine*, **60:**69–90.

Gwozdz JM, Thompson KG, Murray A, West DM, ManktelowBW.2000.Use of polymerase chain reaction assay for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in blood and liver biopsies from experimentally infected sheep. *Australian Veterinary Journal*,**78**: 622-624.

Harris NB and Barletta RG .2001. *Mycobacterium avium* subspecies *paratuberculosis*in Veterinary Medicine. *Clinical Microbiology Review*,**14**(3): 489-512

Hasonova L and Pavlik I.2006. Economic impact of paratuberculosis in dairy cattle herds: a review. *Journal of Veterinary Medicine*,**51**: 193-211.

Hermon-Taylo J.2001. *Mycobacterium avium subspecies paratuberculosis*a cause of Crohn's disease. *Gut*, **49:**755–757.

Ignasi M, Maria R , Ramon J, Juan MG and Saniago L.2002. Paratuberculosis in Free-Ranging Fallow Deer in Spain. *Journal of Wildlife Diseases* ,**38**(3): 629-632

Jessup DA, Abbas B, Behymer D.1981. Paratuberculosis in tule elk in California. *Journal of American Veterinary Medical Association*,**179:**1252-1254.

Judge J, Kyriazakis I, Greig A, Allcroft DJ and Hutchings MR. 2005. Clustering of *Mycobacterium avium* subspecies *paratuberculosis* in rabbits and the environment: how hot is a hot spot? *Applied and Environmental Microbiology*,**71**(10): 6033-6038.

Kennedy DG and Benedictus G.2001. Control of *Mycobacterium avium* subspecies *paratuberculosis* infection in agricultural species,**20**: 151-179.

Khare S, Adams LG, Osterstock J, Roussel A And David L. 2008. Effects of shipping and storage conditions of fecal samples on viability of *Mycobacterium paratuberculosis*. *Journal of Clinical Microbiology*, **46**: 1561–1562.

Kulshrestha RC, Singh J and Chandiramani NK.1980. A study on the prevalence of tuberculosis and Johne's disease in cattle and buffaloes in Haryana state. *The Haryana Veterinarian*,**19**: 139-41.

Kumar S, Singh SV, Singh AV, Singh PK, Sohal JS and Maitra A.2010. Wildlife (*Boselaphus tragocamelus*) small ruminant (goat and sheep) interface in the transmission of'Bison type' genotype of *Mycobacterium avium* subspecies *paratuberculosis* in India. *Comparative Immunology,microbiology and infectitious diseases*,**33**: 145-59.

Lall JM.1963.John's Disease in Cattle, Sheep and Goats. Issue 19 of I. C. A. R. Research Series, Indian Council of Agricultural Research, 1963.

Lawrence WE.1956. Congenital Infection with *Mycobacterium johnei* in Cattle. Veterinary Record, **68**: 312-313.

Lepper AW, Wilks CR, Kotiw M, Whitehead JT and Swart KS.1989. Sequential Bacteriological Observations in Relation to Cell-Mediated and Humoral Antibody Responses of Cattle Infected with *Mycobacterium paratuberculosis* and Maintained on Normal or High Iron In- take. *Australian Veterinary Journal***, 66**(2): 50-55.

Lilenbaum W, Marassi CD and Oelemann WM. 2007. Paratuberculosis: an update. *Brazillian Journal of Microbiology,* 38: 580-90.

Losinger WC. 2006. Economic impacts of reduced milk production associated with epidemiological risk factors for Johne's disease on dairy operations in the U.S.A. *Journal of Dairy Research*,**73**: 33–43.

Mackintosh CG, de Lisle GW, Collins DM, Griffin JF.2004. Mycobacterial diseases of deer. *New Zealand Veterinary Journal*,**52**:163–174.

Manning EJ, Collins MT.2001. *Mycobacterium avium* subspecies *paratuberculosis*: pathogen, pathogenesis and diagnosis. *Revue Scientifique et Technique*,**20**: 133-150.

Manning EJB, Steinberg H, Rossow K, Ruth GR, Collins MT.1998.Epizootic of paratuberculosis in farmed elk. *Journal of American Veterinary Medical Association*,**213**: 1320–1322.

Manning EJ, and Ziccardi M.2000. Johne's disease and captive nondomestic hoofstock: prevalence and prevention. *Proceedings of the American Association of Zoo*

Veterinarians and International Association for Aquatic Animal Medicine Conference: 432-434.

Marsh I, Whittington R, Cousins D (1999) PCR-restriction endonuclease analysis for identification and strain typing of *Mycobacterium avium* subspecies paratuberculosis and *Mycobacterium avium* subspecies *paratuberculosis* based on polymorphisms in IS1311. *Molecular Cell Probes*,**13**:115–126

Mishina D, Katsel P, Brown ST, Gilberts EC and Greenstein RJ.1996. On the etiology of Crohn disease. Proceedings of the National Academic Sciences of USA,***93*:** 9816—9820.

Momotani E, Whipple E, Thiemann A and Gheville N. 1988. Role of M Cell and macrophages in the entrance of Mycobacterium paratuberculosis into domes of ileal Peyer's patches in calves. *Veterinary Pathology*, **25**: 131-137.

Motiwala AS, Amonsin A, Elizabeth MS, Manning JB, Kapur V and Sreevatsan S.2004. Molecular Epidemiology of *Mycobacterium avium* subspecies *paratuberculosis* isolates recovered from wild animal species. *Journal of Clinical Microbiology*,**42**(4): 1703-1712.

Murray RW, Manuel CT, Biljana M.2008. Diagnostic detection methods for *Mycobacterium avium* subspecies paratuberculosis in white-tailed deer. *Canadian Veterinary Journal*, **49**: 683–688.

Musken P, Robino P, Ferroglio E, Rossi L, Meneguz PG, Rosati S.2000. Paratuberculosis in Red Deer (*Cervus elaphus hippelaphus*).*Veterinary Research Communication*,**24**: 435–443.

Naser SA, Schwartz D and Shafran I.2000. Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from breast milk of Crohn's disease patients. *American Journal of Gastroenterology*,**95**:1094-1095.

National Academy of Sciences Report**.** 2003. Diagnosis and control of Johne'sDisease .

National Animal Health Monitoring System .1997. Johne's Disease on US Dairy Operations. Center for Epidemiology and Animal Health, Fort Collins, Colorado

Nebbia P, Robina P, Ferroglio E, Rossi L, Meneguz G and L. Rosati.2000. Paratuberculosis in red deer (Cervus elaphus hippelaphus) in the western Alps. *Veterinary Research Communication*,**24:**435–443.

Nielsen SS, Thamsborg MS, Houe H and Bitsch V.2000. Bulk-Tank Milk ELISA Antibodies for Estimating the Prevalence of Paratuberculosis in Danish Dairy Herds. *Preventive Veterinary Medicine*, **44**(1-2): 297-297.

OnetGE.1997. Current paratuberculosis diagnosis in cattle. *Large Animal Practice*,**18**:18-22.

Pavlik I, Bartl J, Dvorska L, Svastov AP, Du Maine R, Macha ckova M, Yayo AW, Horvathova A. 2000. Epidemiology of paratuberculosis in wild ruminants studied byrestriction fragment length polymorphism in the Czech Republic during the period 1995-1998. *Veterinary Microbiology Journal*,**77**: 231-251.

Pickup RW, Rhodes G, Arnott S, Sidi-Boumedine K, Bull TJ, Weightman A, Hurley M and Hermon-Taylor J.2005. *Mycobacterium avium* subspecies *paratuberculosis* in the catchment area and water of the River Taffin South Wales, United Kingdom, and its potential relationship to clustering of Crohn's disease cases in the city of Cardiff. *Applied and Environmental microbiology*,**71**(4): 2130-9.

Poddoubski IV.1957. La paratuberculose. *Bulletin of the Office of International Epizootics*,**48**: 469-476.

Power SB, Haagsma J, Smyth DP. 1993. Paratuberculosis in farmed red deer in Ireland. *Veterinary Record* ,**132:**213-216.

Pradhan AK, Mitchell RM, Kramer AJ, Zurakowski MJ, Fyock TL, Whitlock RH, Smith JM, Hovingh E, Van Kessel JS, Karns JS And Schukken YH.2011. Molecular epidemiology of *Mycobacterium avium* subspecies *paratuberculosis* in a longitudinal study of three dairy herds. *Journal of Clinical Microbiology*, **49**: 893-901.

Preziuso S, Magi GE and Renzoni G. 2012. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in intestinal and mammary tissues and in lymph nodes of sheep with different techniques and its relationship with enteric lesions. *Small Ruminant Research*, **105**: 295–299.

Rademaker JL, Vissers MM andTeGiffel MC. 2007. Effective heat inactivation of *Mycobacterium avium* subspecies *paratuberculosis* in raw milk contaminated with naturally infected faeces. *Applied and Environmental Microbiology*,**73**(13): 4185-90.

Rahman SK.1985. Clinicopathological studies of Paratuberculosis in sheep and goat and its therapy. Department of Veterinary Epidemiology and Preventive medicine,College of Veterinary Science and Animal Husbandry,OUAT,Bhubaneswar,Odisha,India.

Rideout BA, Brown ST, Davis WC, Giannella RA, Huestan WD and Hutchinson LJ. 2003.Diagnosis and control of Johne'sdisease. *Washington, DC: National Academies Press.*

Riemann H, Zaman MR, Ruppanner R, Aalund O, Jorgensen JB, Worsaae H, Behymer D. 1979. Paratuberculosis in cattle and free-living exotic deer. *Journal of American Veterinary Medical Association*,**174**: 841-843.

Robino P, Nebbia P, Meneguz PG and De Meneghi D .2003. Survey on paratuberculosis in simpatric roe deer (*Capreoluscapreolus*) and small ruminant in North-Western Italy. *Proceedings of 7th Internationall Colloquium on Paratuberculosis*, 472-476.

Robino P, Nebbia P, Tramuta C, Martinet M, Ferroglio E and Meneghi DD.2008. Identification of *Mycobacterium avium* subspecies *paratuberculosis* in wild cervids (*Cervus elaphus hippelaphus* and *Capreolus capreolus*) from Northwestern Italy. *Eur. Journal of Wildlife Diseases Research*,**54**: 357–360.

Rohonczy EB, Balachandran AV, Dukes TW, Payeur JB, Rhyan JC, Saari DA, Whiting TL, Wilson SH, Jarnagin JL.1996. A comparison of gross pathology, histology, and mycobacterial culture for the diagnosis of tuberculosis in elk (Cervus elaphus),**60**: 108-114.

Rowe MT and Grant IR.2006. *Mycobacterium avium* subspecies *paratuberculosis* and its potential survival tactics. *Letters in Applied Microbiology*,**42**(4): 05-11.

Sardana T.2014.Evaluation of 'cattle' and 'Indian Bison' type antigens of *Mycobacterium avium* subspecies paratuberculosis for diagnosis of bovine Johne's disease using 'indigenous ELISA' and AGPT. *Indian Journal of Experimental Biology*,**52**: 1182-1185.

Sarno E, Keller S, Wittenbrink MM and Stephan R. 2013. Occurrence of *Mycobacterium avium* subspecies *paratuberculosis* in fecal samples of hunted deer, chamois and ibex in Switzerland. *Schweizer Archivfur Tierheilkunde*, **155**:523-525.

Schwartz D, Shafran I, Romero C, Piromalli C, Biggerstaff J, Naser N.2000.Use of short-term culture for identification of *Mycobacterium avium* subspecies *paratuberculosis*in tissue from Crohn's disease patients. *Clinical Microbiology and Infection*,**6** : 303-307.

Sechi LA, Scanu AM, Molicotti P, Cannas S, Mura M, Dettori G.2005. Detection and isolation of *Mycobacterium avium* subspecies *paratuberculosis* from intestinal mucosal biopsies of patients with and without Crohn's disease in Sardinia. *American Journal ofGastroenterology,***100**:1529-1536.

Selby W.2000. Pathogenesis and therapeutic aspects of Crohn's disease. *Journal of Veterinary Microbiology* ,**77**: 505-511.

Shah IH, Darzi MM and Mir MS.2012. Comparative Efficacy of Rectal Pinch, Faecal Smear and Faecal Polymerase Chain Reaction Tests for Surveillance of *Paratuberculosis* in Goats (*Capra hircus*). *SKUAST Journal of Research,***14**:17-23.

Sheather AL.1918. Johne's disease. *Indian Journal of Agricultural Sciences India,***13**: 23-31.

Shin SJ, Han JH, Manning EJ and Collins MT.2007. Rapid and reliable method for quantification of *Mycobacterium paratuberculosis* by use of the BACTEC MGIT 960 system. *Journal of Clinical Microbiology* ,**45** : 1941-1948.

Shinnick TM and Good RC. 1994. Mycobacterial taxonomy. *European Journal of Clinical Microbiology and Infecttitous Diseases*, **13**(11): 884-901.

Singh AV, Singh SV, Makharia GK, Singh PK and Sohal JS.2008. Presence and characterization of *Mycobacterium avium* subspecies *paratuberculosis* from clinical and suspected cases of Crohn's disease and in the healthy

human population in India. *International Journal of Infectitious Diseases,**12***: 190-197.

Singh AV, Singh SV, Singh PK and Sohal JS.2010. Genotype diversity in Indian isolates of *Mycobacterium avium* subspecies *paratuberculosis* recovered from domestic and wild ruminants from different agro-climatic regions. *Comparative Immunology, microbiology and infectitious diseases,***33**: 127-131.

Singh SV, Kumar N, Chaubey KK, Gupta S, Rawat KD.2013. Bio-presence of *Mycobacterium avium* subspecies *paratuberculosis* infection in Indian livestock farms. *Research Opinion in Animal and Veterinary Sciences*, **3**:401-406.

Singh SV, Singh AV, Singh PK, Gupta VK, Kumar S and Vohra J.2007. Sero-prevalence of paratuberculosis in young kids using "Bison type', *Mycobacterium avium* subspecies *paratuberculosis* antigen in plate ELISA.*Small Ruminant Research* :1169 .

Singh SV, Singh AV, Singh PK, Singh B, Ranjendran AS and Swain N. 2011.Recovery of Indian Bison Type genotype of *Mycobacterium avium* subspecies *paratuberculosi s*from Wild Bison (*Bosgourus*) in India. *Veterinary Research*, **4** :61-5.

Singh SV, Singh AV, Singh R,Sharma S, Shukla N, Misra S, Singh PK, Sohal JS, Kumar H, Patil PK, Misra P and Sandhu KS.2000. Sero-Prevalence of Johne's Disease in Buffaloes and Cattle Population of North India Using Indigenous ELISA Kit Based on Native *Mycobacterium avium* subspecies *paratuberculosis*'Bison Type' Genotype of Goat Origin. *Comparative Immunology, Microbiology and Infectious Diseases,* **31**(5): 419-433.

Singh AV, Chauhan DS, Singh SV, Kumar V, Singh A, Yadav A and Yadav VS.2016. Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection in animals and humans in India: What needs to be done?.*Indian Journal of Medical Research*, **144:**661-671.

Sockett DC, Conrad TA, Thomas CB and Collins MT.1992. Evaluation of four serological tests for bovine paratuberculosis. *Journal of Clinical Microbiology*,**30:**1134-1139.

Sohal JS, Singh SV, Singh B, Thakur S, Aseri GK, Jain N, Jayaraman S, Yadav P, Khare N, Gupta S, Chaubey KK and Dhama K.2015. Control of paratuberculosis: opinions and practices. *Advances in Animal and Veterinary Science*,**3**(3): 156-163.

Sohal JS, Singh SV, Subhodh S, Singh AV, Singh PK and Sheoran N.2007. *Mycobacterium avium* subspecies *paratuberculosis* diagnosis and strain typing-present status and future developments. *Indian Journal of Experimental Biology*,**45**: 843-852.

Soltys MA, Andress CE, Fletch AL. 1967. Johne's disease in a moose (Alcesalces). Bull. *Wildlife Disease Association*, **3**:183-184.

Sonawane GG, Tripathi BN.2013. Comparison of a quantitative realtime polymerase chain reaction (qPCR) with conventional PCR, bacterial culture and ELISA for detection of *Mycobacterium avium* subspecies *paratuberculosis* infection in sheep showing pathology of Johne's disease. *Springer plus*,**2**: 45.

Sood A, Midha V, Sood N, Bhatia AS and Avasthi G.2003. Incidence and prevalence of ulcerative colitis in Punjab, North India. *Gut*,**52**:1587-90.

Stabel JR, Bosworth TL, Kirkbride TA, Forde RL and Whitlock RH.2004. A simple, rapid, and effective method for the extraction of *Mycobacterium paratuberculosis* DNA from fecal samples for polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation,* **16**:22–30.

Stabel JR.1997. An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. *Journal of Veterinary Diagnostic Investigation*,**9**(4):37580.

Streeter RN, Hoffsis GF, Bech-Nielsen S, Shulaw WP and Rings DM.1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *American Jounal of Veterinary Research*,**56**(10): 1322-4.

Sweeney RW, Whitlock RH, Rosenberger AE.1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *Jounal of Clinical Microbiology*,**30**: 166-171.

Sweeney RW.1996. Collins MT. Transmission of paratuberculosis. *Veterinarey Clinics of North America: Food Animal Practice*,**12**:305-312.

Taya F, Karin O,1 Jeroen DB, SteeveDC,Christine C, Tracy D, Brett E, Allicia K, Martin K,RichardP,Joelle T, Alasdair V,and Susan K.2012. Detectionof *Mycobacterium avium* subspecies *paratuberculosis* in several herds of arctic caribou (*Rangifer tarandus). Journal of Wildlife Diseases*, **48**(4): 918–924.

Temple RM, Muscoplat CC, Thoen CO, Himes EM, Johnson DW. 1979. Observation on diagnostic tests for paratuberculosis in a deer herd. *Journal of American Veterinary Medical Association*,**175**: 914-915.

Twort FW, Ingram GLY.1913. A monograph of Johnes' disease.London: Barilliere, Tindall and Cox.

Ursing B, Alm T, Barany F, Bergelin I, Ganrot-Norlin K and Hoevels J.1982. A comparative study of metronidazole and sulfasalazine for active Crohn's disease: the cooperative Crohn's disease study i+n Sweden. II Result. *Gastroenterology* ,**83**: 550-62.

Vary PH, Andersen PR, Green E, Hermon-Tayler J and McFadden J.1990. Use of highly specific DNA probes and the PCR reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *Journal of Clinical Microbioogy*,**28**: 933-937.

Venugopalen S, Radhakrishnan S, Rajachandrasekharan R.1980.Crohn's disease (a study of 21 cases). *Indian Journal of Surgery*, **42**: 388-96.

Verma DK.2013. *Mycobacterium avium* subspecies *paratuberculosis*: an emerging animal pathogen of Global concern. *Advanced Biomedical Rsearch*,**4**: 1-8.

Von Weber A and Gurke R. 1992. Bakteriologische Untersuchungenzum Vorkommen von *Mycobacterium paratuberculosis* in Kotproben von Damwild [Bacteriological examinations occurrence of *Mycobacterium* paratuberculosis in faecal samples from deer] .*Zeitschriftfür Jagdwissenschaft*, **38**: 55-59.

Von Weber A, Gurke R, Bauer K, Schreyer K. 1992. Bacteriological investigation on the presence of *Mycobacterium paratuberculosis* in fecal samples of zoo ruminants. *Berliner Und Munchene Tierartztliche Wochenscrift* ,**105**:161-164.

Wadhwa A, Hickling GJ and Eda S.2012. Opportunities for improved serodiagnosis of human tuberculosis, bovine tuberculosis, and paratuberculosis. *Journal of Veterinary Medicine International* :674238.

Wells SJ and Wagner BA.2006. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. *Journal of American Veterinary Medical Association*,**216**: 1450-7.

Whan L, Ball HJ, Grant IR and Rowe MT .2005. Occurrence of *Mycobacterium avium* subspecies *paratuberculosis*in untreated water in Northern Ireland. *Applied and Environmental microbiology*,**71**(11): 7107-7112.

Whitlock RH and Buergelt C.1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). *Veterinary clinics of north America:Food animal practice.***12:**345–356.

Whittington RJ, Marsh IB andReddacliff LA.2005. Survival of *Mycobacterium avium* subspecies *paratuberculosis* in dam water and sediment. *Applied and Environmental Microbiology*,**71**(9): 5304-5308.

Whittington RJ, Sergeant ESG. 2001. Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. *Australian Veterinary Journal*, **79**:267-278.

Williams ES.2001. Paratuberculosis. *In E. S. Williams and I. K.Barker (ed.), Infectious diseases of wild mammals, 3rd ed. Iowa State UniversityPress, Ames, IA*: 361–371.

Windsor PA, Eppleston J, Sergeant E. 2003. Monitoring the efficacy of Gudair. TM OJD vaccine in Australia. *Proceedings of Australian Sheep Veterinary Society*,**13**:114-122.

Yokomizo Y, Merkal RS and Lyle PA.1983. Enzyme- Linked Immunosorbent Assay for Detection of Bovine Immunoglobulin G1 Antibody to a Protoplasmic Antigen of *Mycobacterium paratuberculosis.American Journal of Veterinary Research*, **44**(11):2205- 2207.